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EFFECT OF BASIC PROTEIN FROM TUMOR TISSUE ON LIVER TRYPTOPHAN PYRROLASE ACTIVITY

TAKASHI KAWACHI, SETSURO FUJII, TOMOKAZU SUZUKI, NORIO UESAKI and YUICHI YAMAMURA
(Department of Biochemistry, School of Medicine, Kyushu University)

It has been reported that the basic protein from tumor tissues possesed toxohormone activity (1). The basic protein was capable of decreasing liver catalase activity and plasma iron level when it was injected intraperitoneally into animals. In the action of toxohormone, Fukuoka and Nakahara (2) has presented the hypothesis that toxohormone might suppress the synthesis of liver catalase by impairing the utilization of iron. The effect of toxohormone on the porphyrin metabolism was studied by Ono *et al.* (7) and there has been reported that the considerable increase of protoporphyrin contents in liver and of coproporphyrin excretion in the urine of tumor bearing animals. The similar tendency of the porphyrin metabolism was observed in normal animals by the injection of toxohormone fraction. These results indicated that the underutilization of porphyrin in tumor bearing animals was caused by the impairment of iron metabolism due to the activity of toxohormone. Tryptophan pyrrolase contains porphyrin as prosthetic group and it was assumed to be an adaptive enzyme found in mammalian liver. The activity of tryptophan pyrrolase was greatly increased after injection of tryptophan (3).

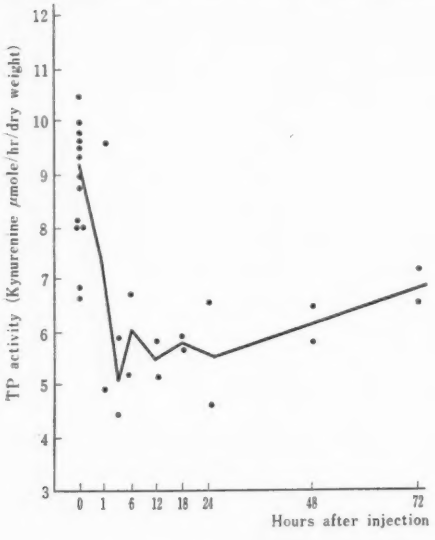
In this report, the effect of the basic protein on liver tryptophan pyrrolase activity was studied.

EXPERIMENTALS

The basic protein was obtained from Rhodamine sarcoma by the procedure described previously (1). Livers of female Wistar-King rats weighing 150–200 g. were used in enzyme estimation. The basic protein was dissolved into 0.5 ml. of physiological saline, adjusted to pH 7.0 by the addition of 1 N NaOH and injected intraperitoneally at various doses into rats. The animals were killed by decapitation at appropriate intervals after injection. The liver was quickly removed and tryptophan pyrrolase activity was measured by the spectrophotometric determination of the amount of kynurenine formed during aerobic incubation (4). One gram of liver was removed and homogenized in 7 ml. of 0.14 M KCl solution containing 0.0025 N NaOH. One ml. of homogenate was pipetted and transfered into the incubation flask containing 1 ml of 0.2 M phosphate buffer pH 7.0, 0.3 ml. of 0.03 M L-tryptophan (replaced by

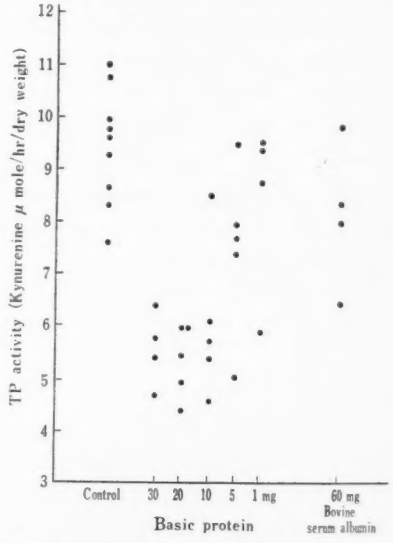
water in the blank) and 1.7 ml. of water. The homogenate was added last, after preliminary temperature equilibration of the other components. The reaction mixture was shaken at 38° in an atmosphere of O_2. One hour after addition of the homogenate, the reaction was stopped with 2.0 ml. of 15 per cent metaphosphoric acid. Then the reaction mixture was filtered. Three ml. of the reaction filtrate was adjusted to pH 6.5 to 7.5 by the addition of 1.0 N NaOH. The optical density of this solution was read against water at the wave length of 365 mμ in a Beckman spectrophotometer. The amount of kynurenine formed was calculated from the optical density of the experimental filtrate minus its blank, by the use of the molar extinction coefficient of kynurenine under these conditions; e=4540 (1 mole per liter, 1 cm. depth.) Dry weight, less the added KCl, was determined from 1 ml. of homogenate dried for 2 or 4 hours at 110°.

The activity of the homogenate was expressed as the number of moles of kynurenine formed per gm. of dry weight per hour. In each assay, the control animals injected with 0.5 ml, of physiological saline were employed.



Female Wistar-King rats weighing 150–200 g. were injected intraperitoneally with 20 mg. of the basic protein and the liver TP activity was measured at various periods after injection.

Fig. 1 The effect of the basic protein isolated from Rhodamine sarcoma on the liver TP activity at various periods after intraperitoneal injection.



Female Wistar-King rats weighing 150–200 g. were injected intraperitoneally with the basic protein in various doses and TP activity was measured at 16 hours after injection.

Famale Wistar-King rats weighing 150–200g. were injected with 20 mg. of bovine serum albumin per day for 3 days

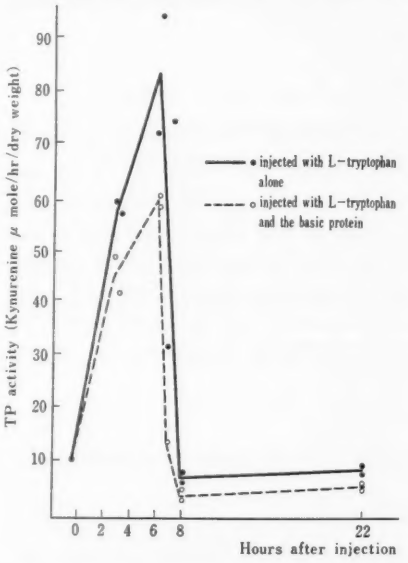
Fig. 2 The effect of the basic protein from Rhodamine sarcoma or bovine serum albumin on the liver TP activity in various doses.

Results

Tryptophan pyrrolase (TP) activity was measured at various intervals after the injection of 20 mg. of the basic protein. As shown in Fig. 1, TP activity decreased to one-half of the normal level at 3 hours after injection. The decrement of enzyme activity continued for more than 24 hours, and its activity did not recover to the normal level within 72 hours.

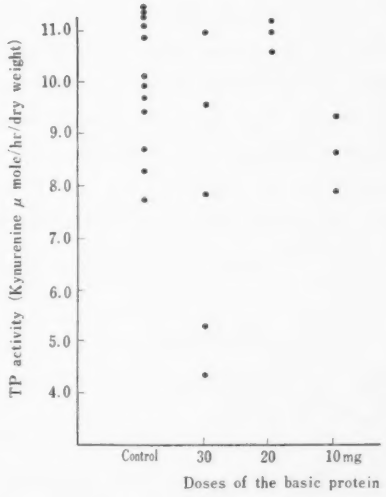
The effect of the basic protein on the liver TP activity was tested in various doses at 16 hours after injection and the result was given in Fig. 2. In order to obtain one-half reduction of the liver TP activity, 10 mg. of the basic protein was required. Twenty mg. of bovine serum albumin was incapable of decreasing TP activity after repeated injection for three days.

Liver TP activity increased about ten folds as much as normal level by intraperitoneal injection of 100 mg. of L-tryptophan per 100 g. of rat body weight. When L-tryptophan and the basic protein were injected at the same time, the extent of



Famale Wistar-King rats weighing 150 to 200 g. were injected intraperitoneally with L-tryptophan (100 mg. of L-tryptophan per 100g. of rat body weight) or L-tryptophan and 20 mg. of the basic protein. The liver TP activity was measured at various periods after injection.

Fig. 3 The effect of the basic protein isolated from Rhodamine sarcoma on the adaptive increment of the liver TP activity.



Female Wistar-King rats weighing 150-200 g were injected intraperitoneally with the basic protein in various doses and TP activity was measured at 16 hrs. after injection.

Fig. 4 The effect of various doses of the basic protein isolated from liver of normal rat on liver TP activity.

induction was less than that in the case injected with L-tryptophan alone. Reduction of adaptive increment of TP activity by the basic protein was presented in Fig. 3. A 30% of reduction was observed at any time during the experiment.

The effect of the basic protein on the liver TP activity was tested *in vitro*. One gram of liver of normal rat was removed quickly after decapitation, added with 7 ml. of 0.14 M KCl solution and homogenized. As a control experiment, one ml of the homogenate was pipetted, added with 1 ml. of 0.2 M phosphate buffer (pH 7) and 1.7 ml. of the distilled water. In the experiment to test the effect of the basic protein, 1.7 ml. of the distilled water containing 12.5 mg, 1.25 mg and 0.125 mg of the basic protein respectively, was added to the homogenate instead of distilled water and the mixture was subjected to the determination of TP activity immediately or after incubation at 0° for 60 min. As seen in Table 1, the basic protein did not inhibit TP activity *in vitro* under these conditions at various concentrations.

As control experiment, the basic protein was isolated from normal rat liver by the same method described above and injected intraperitoneally into rat. The basic protein of normal rat liver had no effect on the liver TP activity of rat even when 30 mg of the basic protein was injected into rat.

Discussion

Nakahara (6) and Greenstein (5) showed that toxohormone had a marked inhibitory effect on liver catalase *in vivo* but not *in vitro*. It has been reported from our laboratory that the basic protein isolated from tumor tissues also decreased liver catalase activity *in vivo* and not *in vitro*. As shown in this report, decrease to less than half the normal level of TP was also observed in the liver of rat injected with the basic protein and this inhibitory effect on enzyme was not found *in vitro*

Table 1. The effect of the basic protein from Rhodamine sarcoma on the TP activity *in vitro*.

Preincubation time at 0° \ Basic protein added		0	12.5 mg	1.25 mg	0.125 mg
TP activity (Kynurenine γmole/hr/dry weight)	0 min.	8.50	7.43	7.03	8.2
	60 min.	8.02	7.03	7.00	7.6

The experimental condition was described in the section of "Results."

experiments. Further, the extent of induction of TP by administration with tryptophan was reduced by the injection of the basic protein. On the basis of an assumption that this adaptive increment of TP activity is induced by the increase of biosynthesis of the enzyme, it is suggested that the decrease of TP level by injection of the basic protein is due to the inhibitory effect on the new formation of the enzyme.

The decrease of liver catalase activity caused by the injection of toxohormone was prevented partially by the administration of ferric chloride (2) and the injection of toxohormone showed the increase of protoporphyrin contents in normal rats (7). From these facts, Fukuoka and Nakahara presented the hypothesis that toxohormone might suppress the synthesis of the liver catalase by imparing the utilization of iron. As TP contains iron in its chemical configuration, the fact that this enzyme level is reduced by the basic protein might support the hypothesis suggested by Fukuoka and Nakahara.

Further studies on the mechanism of effect on TP, catalase and plasma iron level by the basic protein is needed.

Summary

1. The effect of the basic protein isolated from tumor tissues on the liver TP activity of normal rat was examined.

2. Administration of the basic protein reduced TP activity. The minimum effective dose was 10 mg.

3. The adaptive increment of TP activity with L-tryptophan was also reduced by the administration of the basic protein.

4. The basic protein did not inhibit TP activity *in vitro*.

5. The basic protein isolated from the liver of normal rat had no effect on TP activity even when 30 mg of the basic protein was injected into rat.

Acknowledgment

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STUDIES ON TRYPTOPHAN PYRROLASE OF TUMOR-BEARING RATS WITH SPECIAL REFERENCE TO THE FUNCTION OF ADRENAL GLANDS

TAKASHI KAWACHI. SETSURO FUJII, NORIO UESAKI, TOMOKAZU SUZUKI and YUICHI YAMAMURA

(Department of Biochemistry, Faculty of Medicine, Kyushu University)

Tryptophan pyrrolase (TP) is a characteristic enzyme in mammalian liver. The activity is greatly influenced by two mechanisms. The first mechanism in response to the administration of L-tryptophan is the adaptive increment of the enzyme activity (1). The second is a hormonal regulation of the metabolism. The activity is moderately increased after the injection of L-tyrosine, L-histidine, histamine and epinephrine (2) that are thought to have stress action through the adrenal glands (3). Cortisone, hydrocortisone (4) and adrenocorticotropic hormone (5) increase TP activity, while growth hormone (6), hypophysectomy (7) and adrenalectomy (3) decrease TP activity.

It was reported previously that the basic protein isolated from tumor tissues decreased plasma iron level, liver catalase activity (8), TP activity and adaptive increment of TP activity (9). In the tumor-bearing rats, liver catalase activity (10) and plasma iron (11) showed a marked decrease.

In this report, it was shown that TP activity of the tumor-bearing rats was presented to be on the balance of the toxohormone activity produced by the tumor tissues and of the stress reaction through the adrenal glands.

EXPERIMENTALS

Japanese mixed strain rats weighing 150–250 g were transplanted with Rhodamine sarcoma, and the activity of liver TP, catalase and plasma iron level were determined at various periods after the inoculation of tumor. The adrenalectomy of tumor-bearing rats was performed bilateraly without anesthesia two weeks after the tumor transplantation. The tumor-bearing rats which were adrenalectomized had a high mortality of about 50%, while the adrenalectomized rats without tumor survived for a long time under good care. When tumor-bearing rats were adrenalectomized four weeks after transplantation of tumor, the operation caused the death in all cases. In our experiments, the TP activity was measured on the 7th day after adrenalectomy.

Assays - The animals were killed by decapitation and the livers were removed promptly. TP activity was determined by the method of Knox (12). Liver catalase

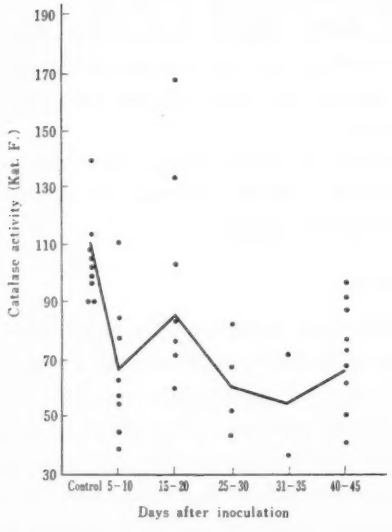
assay was carried out by the modified method of von Euler and Josephson (13). Plasma iron level was measured by the method of Barkan and Walker (14).

In experiments to test the effect of the basic protein from tumor tissue on the liver catalase activity of adrenalectomized animal, ddN strain female mice (one month old) were used. The basic protein was isolated from Rhodamine sarcoma by the method previously reported (8). Five mg of the basic protein were injected intraperitoneally into normal and adrenalectomized mice. The activity of liver catalase was measured at 24 hours after injection.

Results

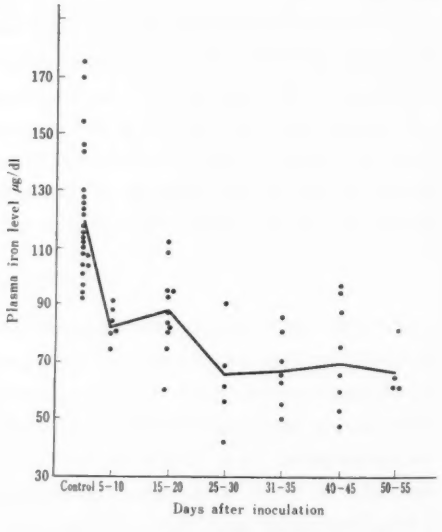
Changes of liver catalase activity, TP activity and plasma iron level after the inoculation of Rhodamine sarcoma are shown in Figs. 1, 2 and 3. The weight of tumor 5~10 days after inoculation was 1~4 g. As seen in Figs. 1 and 2, liver catalase activity and plasma iron showed a remarkable decrease in 5~10 days after inoculation and the decrease continued for the whole period of experiment.

On the other hand, as shown in Fig. 3, TP activity of tumor-bearing rats showed various levels. In early phase of tumor growth, TP activity tended to decrease. Thereafter, the activity of enzyme showed various levels, while normal rat had a



Japanese mixed strain weighing 150-250 g were transplanted with Rhodamine sarcoma and the liver catalase activity of rats was measured at various periods after inoculation.

Fig. 1 Changes of liver catalase activity after the inoculation of Rhodamine sarcoma.



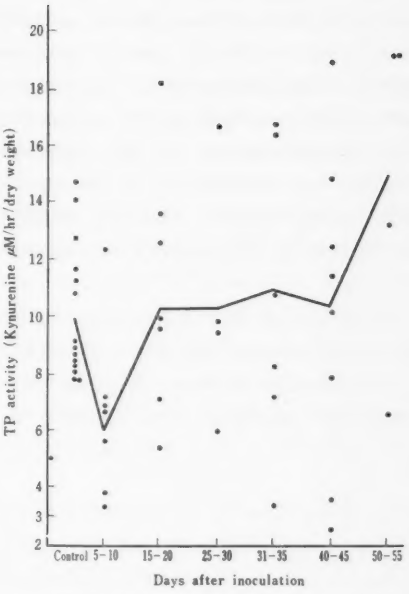
Japanese mixed strain weighing 150-250 g were transplanted with Rhodamine sarcoma and the plasma iron level of rats was measured at various periods after inoculation.

Fig. 2 Changes of plasma iron level after the inoculation of Rhodamine sarcoma.

fairly constant level of TP activty.

There was a critical point after 15~18 days of tumor growth, at which time the enzyme level changed from the early low level to late various levels. The relationships of liver catalase activity, plasma iron level and TP activity were shown in Figs. 4, 5 and 6. A good correlation was found between catalase activity and plasma iron level, indicating the changes of catalase and plasma iron level were caused probably by the same factor, toxohormone. Consequently, the tumor-bearing animals which had relative normal value of catalase activity showed normal level of plasma iron, and animals which had low value of catalase showed low level of plasma iron. No correlation was obverved between catalase activity or plasma iron level and TP activity.

TP activity of tumor-bearing mice was investigated by Knox *et al.* (6) and Ichii



Japanese mixed strain weighing 150-250g were transplanted with Rhodamine sarcoma and the liver TP activity of rats was measured at various periods after inoculation.

Fig. 3 Changes of liver TP activity after the inoculation of Rhodamine sarcoma.

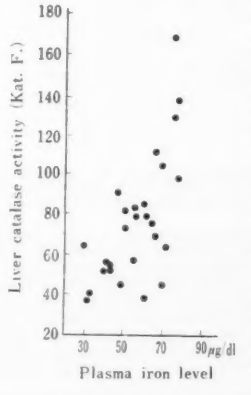


Fig. 4 Correlation of liver catalase activity and plasma iron level of tumor bearing rats.

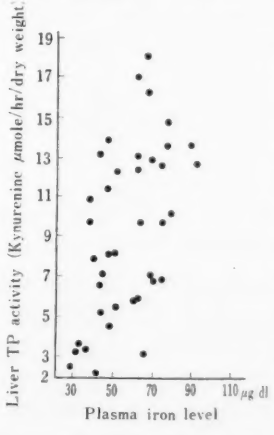


Fig. 5 Correlation of liver TP activity and plasma iron level of tumor bearing rats.

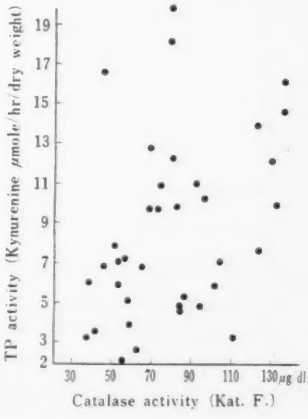
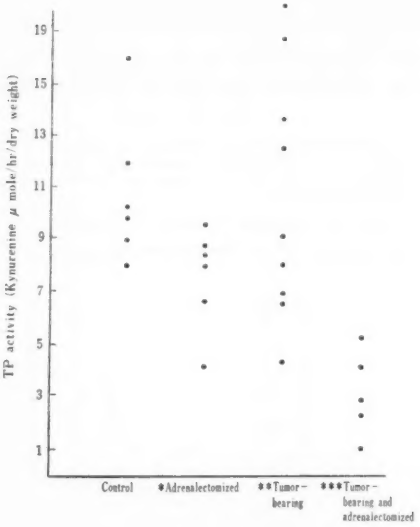


Fig. 6 Correlation of liver TP activity and liver catalase activity of tumor bearing rats.

(15). They reported that depression of the enzyme activity to one-half of the control level was found in mice during the initial period of tumor growth, and after the first 18 days of tumor growth, significant increase in the enzyme activity was observed. The increment of average value of TP activity in late period of tumor growth was not found in this experiment, showing that average of enzyme activity was around normal. It was reported previously by Kawachi *et al.* (9) that TP activity was decreased by the intraperitoneal injection of the basic protein isolated from tumor tissues. This fact might suggest that the decrement of TP activity of the tumor-bearing animals was caused by toxohormone in whole period of growth of tumor.

In connection with TP activity in late period, Wood *et al.* suggested that the increment of this enzyme was due to the influence of adrenal hormone stimulated by development of tumor from the fact that TP activity was sensitive to hormonal regulation especially glucocorticoid (7). Thus the determination of TP activity in



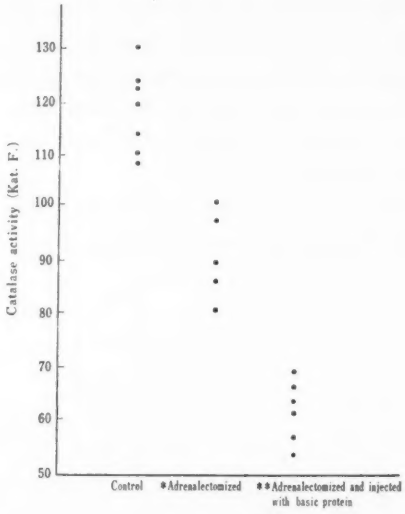
Japanese mixed strain rats weighing 150-250 g were used

* Normal rats were adrenalectomized bilateraly and liver TP activity was measured on the 7th day after operation.

** Liver TP activity was measured at three weeks after transplantation of Rhodamine sarcoma.

*** Tumor bearing rats at 2 weeks after transplantation were adrenalectomized bilateraly and liver TP activity was measured on 7 days after operation.

Fig. 7 The effect of adrenalectomy on liver TP activity of tumor bearing rats.



Female ddN strain mice (one month old) were adrenalectomized bilateraly without anesthesia.

* andrenalectomized mice were measured on 10 days after operation.

** 5 mg of the basic protein was injected intraperitoneally into the adrenalectomized mice on 10 days after operation and liver catalase activity was measured at 24 hr after injection.

Fig. 8 The effect of the basic protein from tumor tissues on liver catalase activity of the adrenalectomized mice.

tumor-bearing adrenalectomized animals might give more precise evidence that late elevation of enzyme is due to the effect of adrenal hormone. The result is shown in Fig. 7. TP activity of adrenalectomized rats decreased to two-third the control rats. When the tumor-bearing rats were adrenalectomized after three weeks of tumor inoculation, TP activity of the rats showed a marked decrease in all cases and the degree of decrease of enzyme level in tumor-bearing animals was greater than that of the adrenalectomized rat without tumor.

Administration of cortisone on normal animals did not affect liver catalase activity, while the activity was decreased by adrenalectomy and the injection of cortisone to adrenalectomized mice restored the catalase activity to normal level (15). The effect of the basic protein on liver catalase activity of the adrenalectomized mice was shown in Fig. 8. Liver catalase activity was decreased to two-third the control mice by adrenalectomy. The basic protein decreased liver catalase of adrenalectomized mice to one-third the control mice in all cases. The data obtained showed that the decrement of catalase activity by toxohormone was brought about without the mediation of adrenal gland influence.

From the point of view of rapid growth and remarkable development, tumor tissue and fetus have the same charactor. Consequently, the liver TP activity of the pregnant animals was determined using Wister-King rats on about 20th day after conception. As shown in Table 1, the TP activity of pregnant rats liver was in normal level.

Table 1. Liver TP activity of the pregnant rats.

	Number of animals	Liver TP activity (μmoles Krynurenine/ hr/g. dry weight)
Normal rats	20	10.63±2.0
Pregnant rats	3	9.85±3.54

Pregnant rats of Wistar-King strain were used 20 days after conception in this experiment.

Discussion

It was reported by Wood *et al.* (6) that TP activity of tumor-bearing mice had a tendency to show biphasic change of depression and elevation. However, as seen in Fig. 3, our data were not so clear as Wood *et al.* In early phase, the activity was depressed and in late period, TP activity showed not only high level, but also low level. Average value of TP activity in late period was around normal. When tumor-bearing rats were adrenalectomized, TP activity showed striking decrease without exception. Wood *et al.* suggested that the early depression was due to growth hormone (6), while it was reported by Kawachi *et al.* that administration of the basic protein from tumor tissues decrease TP activity of normal rats (9), indicating that

the depression of liver TP activity in early phase was due to the presence of some toxic substances produced in tumor tissues. It was suggested in this report that the late high level of TP activity was the result of stress reaction of adrenal glands.

Toxohormone isolated from tumor tissues was capable of decreasing liver catalase activity when injected into normal animal (17). It was not known whether the action of toxohormone and basic protein from tumor tissues was direct or indirect, e.g., *via* glands such as adrenal and hypohyseal glands. Our results, as seen in Fig. 8, suggest that the basic protein decreased liver catalase activity with no correlation to the function of adrenal glands.

Summary

1. Liver catalase activity and plasma iron level showed a remarkable decrease in tumor-bearing rats.

2. In early phase of tumor inoculation, liver TP activity of tumor-bearing rats tended to decrease the level, thereafter, the activity showing various levels.

3. TP activity of tumor-bearing rats was decreased strikingly by adrenalectomy.

4. The basic protein decreased liver catalase activity of adrenalectomized mice.

5. TP activity of pregnant rats was in normal level.

6. TP activity of tumor-bearing rats and its relation to the adrenal glands were discussed.

Acknowledgment

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HISTOPATHOLOGICAL STUDIES ON TRANSPLANTED EXPERIMENTAL OSTEOGENIC SARCOMA

(Plates XV—XVII)

KENYA HORIE, MOTOO MAKITA and KAZUTO SATO
(Tachikawa Hospital, Tachikawa, Tokyo)

The experimental production of osteogenic sarcoma in tibia of albino rats by administering various carcinogenic substances in combination with radioisotope P^{32} with discussions on its histogenesis was previously reported[1]. This sarcoma has now been successfully transplanted for successive generations into albino rats of the same strain.

There are many reports on the production of experimental osteogenic sar-coma, but we have not seen any report of success in successive transplantations. The fate of this osteogenic sarcoma during the transplantations was followed histopathologically and observations were made on the effects of administering substances which may affect the sarcoma such as X-ray radiation, various anticancerous drugs, Predonin and AAN, in an effort to clarify the character of the tumor.

Experimental Method and Material

An osteogenic sarcoma produced by administering P^{32} in combination with carcinogenic substances in albino rats of wistar strain was cut into small pieces and transplanted into the backs of albino rats of the same strain subcutaneously. We succeeded in transplanting the sarcoma successively from one generation to another. This successively transplanted sarcoma was studied pathologically, both grossly and microscopically, and its fate was followed. Further, the effects of X-ray radiation, anticancerous drugs (Nitromin, Tespamin, Mitomycin C, Carzinophilin, Sarkomycin, and Azan), Predonin, and AAN on the transplanted tumor was studied histopathologically.

The albino rats with the transplanted sarcoma were given the above substances in the quantities of 1/10 of LD 50. Among the anticanceroue drugs Nitromin and Tespamin are known as cytotoxic and Azan as antimetabolic substances. Mitomycin C, Carzinophilin, and Sarkomycin are antibiotics. Predonin has inhibitory action on protein metabolism. AAN is a synthetic lathyrus factor which interferes with bone formation and maturation and produces exostoses. The administration of these substances was started after making certain that the tumors had grown to a certain size (2 cm^3).

Results

When the sarcoma is successfully transplanted, it appears grossly as shown in Figure 1. The shape is unevenly round and protrusions are noted on the surface. The skin covering the tumor loses fur as the tumor enlarges and often portions become necrotic and form ulcers. The consistency is firm and elastic like cartilage. Many tumors are lightly adherent to the skin and the underlining tissue, but the border is clearly distinguishable.

There is slight resistence on blunt dissection of the tumor. The dissected tumor is grayish white, lustrous white, or pinkish in colour.

The cut surface often appears grayish white and lustrous. Hemorragic and necrotic foci are secn in larger ones. As the tumor increases in size, the general condition of the sarcomatous animal becomes weak, the fur loses luster, falls out, and becomes thin. Terminally the animal becomes anemic, cachectic, and dies. The rate of successful transplantation is approximately 42% (176/417) during successive transplantations to the 12th generation, which has been reached at the time of this writing.

Changes in the sarcoma change during the course of transplantation.

By allowing the tumor to run its natural course, 3 different cases are noted:

1) There are tumors which show almost no change in the histopathological picture after being successfully transplanted from generation to generation.

The degree of osteoplasia of this primary experimental osteogenic sarcoma varies and on transplanting small pieces of tissue from these sarcomas of vaious degrees of osteoplasia it is noted that the transplantations made with tissues with high degree of osteoplasia, that is well differentiated tissues, do not take, while those made with tissues with low degree of osteoplasia, that is undifferentiated tissues, are successful. These undifferentiated sarcomatous tissues, as previously reported, are found more often in the periphery of the tumor.

Once the tumor starts to grow and increase in size, it seldom is absorbed or disappears spontaneonsly. In most cases it continues to grow until the death of the animal. However, metastases have not yet been observed.

These transplanted sarcomas show histopathological picture of immature osteogenic sarcoma identical with the original primary osteogenic sarcoma. As shown in Figure 2, the histological pattern of this tumor is composed chiefly of immature cell groups. The cells with slight variation are medium in size. The nucleus is round or oval in shape and stains darkly. The cytoplasm is basophilic and small in amount. The cells are closely packed together and the cell outline is not clear.

Two types of tissue can be discerned in the tumor. One type is made up of the above mentioned tumor cells arranged both massively and diffusely together and

shows tissue pattern similar to an ordinary sarcoma.

The other type is composed of blood vessels and tumor cells intimately arranged together. As seen in Figure 3, the tumor cells form lobules or funiculi. Between these groups of cells are often noted blood sinuses or loose connective tissue. This tissue pattern seen in the latter type is often observed in the primary experimental osteogenic sarcoma.

Further, in the case of the primary sarcoma, deposition and formation of bone matrix, a process in bone formation, can be followed. Consequently it can be said that the histological picture of the latter, even though it may be in the stage which does not show bone matrix, that of an immature osteogenic sarcoma.

2) There are tumors in which osteoplasia, fibrosis, or giant cell tumor like histological picture is observed. It has already been mentioned that in this subcutaneous transplantation experiment, those made with immature sarcomatous tissue without osteoplasia are the successful ones. However, this sarcoma in some cases does not remain immature and shows tendency toward fibrosis (Figure 4). At times it may act as a fibrotic tumor and again revert to an osteoplastic tumor (Figure 5). And at other times it may show many giant cells as seen in the clinical type of giant cell tumor (Figure 6). However, even in such cases, the pattern of blood sinuses remains, indicating the osteogenic origin of the tumor.

3) There are tumors which atrophy with each repetition of transplantation and finally are absorbed and disappear. In some series tumor cells begin to atrophy and within a few generations the tumor cells disappear complately. The following is a report of such a series. The first generation, as shown in Figure 7, shows a histological picture of an immature sarcoma.

The second generation is almost identical with the first showing no change.

In the third and fourth generations the histological picture is almost the same as the original primary tumor but groups of atrophic cells can be seen in some portions. These atrophic cells are long and narrow when compared with the cells of the primary tumor. The cells become dissociated and the intercellular spaces increase. In the fifth generation, as shown in Figure 8, the atrophy becomes stronger, vacuolization being conspicuous and intercellular spaces increasing. These changes are no longer localized but are spread diffusely and inhibit the proliferation of the tumor cells. Finally in the sixth generation the transplanted piece of tissue was absorbed and the transplantation did not succeed.

Effects of X-ray and various drugs on the transplanted sarcoma.

The transplanted sarcomas in most cases show a definite histological pattern, as described above, but at times changes in this pattern were noted. What are the factors which cause changes in the pattern? Also what effects the therapeutical

drugs have on this sarcoma becomes an interesting subjects. The effects of X-ray radiation on tissues varies with the quantity and the method of radiation, and on the variety and condition of the tissues radiated. After a single dose of a relatively small amount of 250r very slight edema only is noted, but difinite effect is observed with 1500r (in 3 daily doses of 500r each).

In the case which was radiated with 2000r (in 4 daily doses of 500r each), the animal died the following day. Although degeneration and atrophy are noted histologically in these cases, they are not conspicuous grossly. Figure 9 is a histological picture of the tumor radiated with 250r and shows some dissociation of cells and small amount of edematous fluid is noted in the intercellular spaces. Foci of necrosis are not observed. Variation in size of the tumor cells, mononuclear and polynuclear giant cells, and very slightly atrophied cells scattered throughout are noted.

In the case of 1500r radiation (in 3 daily doses of 500r each), as show nin Figure 10, intense atrophy and dissociation of the tumor cells are noted. The cytoplasm is small in amount and the nuclei become elongated. Thus the atrophy of the tumor cells are prominent, and the appearance of the mononuclear and polynuclear giant cells becomes conspicuous. Besides the above findings there are foci of necrosis, which, when compared with those caused by the administration of anticancerous drugs, do not have clear cut margins and change gradually over into ordinary sarcomatous tissue. Products of decomposition, such as fragmenis of broken down cell nucleus are not noticeable. The necrotic protions in most cases are hyaline.

Next 6 anticancer drugs were administered to these sarcoma transplanted animals, cytotoxic, antimetabolic, and antibiotic substances, having different anticancerous action, were administered in order to observe the difference in effect on this sarcoma. The anticanceous drugs tested were : nitromin (8 mg/kg/day), tespamin (1.5 mg/kg/day), carzinophilin (1500 units/kg/day), sarcomycin (150 mg/kg/day), mitomycin C (0.3 mg/kg/day) and azan (15 mg/kg/day), which were administered intraperitoneally daily for 7 consecutive days. Experiments, carried out with 50 animals in all, showed little or no difference in the histological effects among the drugs used. Therefore the histological findings of a case in which tespamin was administered will be described as an example.

As shown in Figure 11, the distinctive feature is the foci of necrosis which are relatively localized. In the necrotic area loss of nuclei of the cells and remains of the decomposition of nuclei and cytoplasm are observed. Around the preiphry of this necrotic focus there is a narrow zone of degeneration and on the outter side of this there exists atrophied sarcomatous tissue. Within this zone of degeneration giant cells are somewhat conspicuous.

When predonin is administered intramusculasly to the sarcomatous animals in 20 daily doses of 50 mg/kg each, the growth of the tumor is considerably inhibited.

The tumors become larger than prior to the administration of predonin, but when compared with the controls, they are about one-fifth in size. Also there were some transplants which atrophied and disappeared. On examining them histologically the tumor cells are elongated and atrophied, but the giant cells remained.

As the tumor cells atrophy, collagen fibers and silver staining fibers increase. The histological picture takes on the appearance of a fibrosarcoma accompanied by giant cells. The necrotic foci are much less in number when compared with those of X-ray radiated group or those treated with anticancerous drugs. Predomin and AAN combinedly administered group shows almost the same findings as the above predonin group. In the AAN administered group, the clinical findings on the experimental animals were anorexia, loss of weight, and coarseness of fur. The animals become weak but the tumor continues to grow similarly as in the controls. The histological picture also does not differ much from that of the controls.

Discussion

Transplantations of osteogenic sarcomas have been attempted many times, but we are not aware of any report of success in serial retransplantations. The authors studied the histogenesis of experimental osteogenic sarcoma they produced and noted that the immature sarcomatous tissue exists mostly in the periphery of the tumor, and by using small pieces of this immature tissue in the transplantation into the subcutaneous tissue of the back of albino rats, they successfully accomplished serial transplantations of the osteogenic sarcoma.

What will happen to this transplanted sarcoma if transplantation was repeatedly continued? As already stated serial transplantations of this osteogenic sarcome can be repeatedly continued if the immature sarcomatous tissue is used for transplantation. On examining many of these transplanted tumors, they can be classified in general into three groups:

In the first group are the tumors which on being transplanted do not change and continue the immature form of the previous tumor. The histological picture is almost unchanged from that of the primany tumor, and the pattern of the close relation of the tumor cell groups and the blood vessels remain. This type can be utilized for therapeutic experiments.

In the second group are the tumors which have little tendeney toward ossification and greater tendency toward fibrosis. The tendency for these differentiations increase toward the center. As shown in Figure 5, at times during the course of fibrosis ossification may occur. At other times giant cell tumor like histological picture, containing many giant cells, are seen, as shown in Figure 6. These fiindings which appear at a glance as those of fibrosarcoma clearly verify the fact that this tumor originates from the immature bone.

In the third group are tumors which disappeared in a few generations.

There are some series in transplantation in which the tissue degenerated and atrophied and the transplated tumors were absorbed and disappeared.

It is probable that in these cases the transplanting pieces of tissue were taken from a portion with lowered proliferating ability.

The relation of osteogenic sarcoma and fibrosarcoma will be discussed in view of the fact that in this sarcoma only a small amount of osteoplasia and relatively more fibrosis is present. Concerning fibrosarcoma of the bone, its place in the classification is not definite even now. That is to say, even if this fibrosarcoma is found in the bone, can it be said that it orginated in the bone? Further, even if it does originate in the bone, can it be said that it will not change into osteoplastic sarcoma somewhere in the course of its evolution?

Jeffe[2] has classified fibrosarcoma definitely from the clinical pathological view point and considered it as a distinct tumor entity. Concerning the term "osteogenic sarcoma," he defines it as a "malignant connective tissue sarcoma which actually form neoplastic osteoid and osseous tissue in the course of its evolution." Thus he uses the term in a more limited sense to mean sarcoma which can prove its origin in the bone by evidence of osteogenesis rather than to mean one which originates in the bone. Also he places fibrosarcomas of bone into two groups of well differentiated and poorly differentiated, and considers it very difficult to distinguish the latter from osteolytic osteogenic sarcoma. These facts indicate that to determine the origin of an anaplastic sarcoma is exceedingly difficult. Furthermore, to find appropriate terminology for sarcomas which originate in immature bone tissue and which do not form bone matrix but show fibrosis only is exceedingly difficult. The authors proved the existence of the osteogenic fibrosarcoma experimentally, without taking the clinical aspects into consideration.

This sarcoma in the future in certain environment or under certain conditions may manifest fully its potential capacity of osteoplasia. It is possible that, when the transplantation is made into subcutaneous tissue, it is unable in such environment to manifest its osteoplastic potential capacity fully and only fibrosis which is one constituent of bone matrix appears conspicuous. Furthermore, it raises an interesting question of what changes will occur if various influences were to be exerted on this transplanted sarcoma. Consequently various substances were made to act upon this sarcoma and the changes in the sarcomatous tissue were observed. Some differences in effects on the sarcoma were noted in the actions of X-ray radiation, anticancerous drugs, and predonin. In the case of X-ray radiation, if given in small doses, only edema was noted, but in larger doses atrophy and hyaline necrosis were present. Also, the appearance of giant cells and variation in size of the cells were conspicuous. In the case of anticancerous drugs, although the drugs administer-

ed have various different modes of action, the difference in effects were not evident, The distinctive features here were circumscribed areas of necrosis, pyknotic nucleus, karyorrhexis, and karyolysis. Generalized atrophy is also noted. In other words the difference between the effects of the X-ray radiation and the anticancerous drugs is that the former is diffuse while the latter is relatively circumscribed, which probably is due to the fact that the anticancerous drugs are transpoted into the sarcoma by the circulation, causing somewhat uneven distribution, while in the former the unevenness is due solely to the susceptibility of the cells of the tumor tissue to radiation and therofore the changes are relatively more diffuse.

In the case of predonin administration, the necrotic changes were scanty, the tissue atrophying and taking the course of hyalinization and fibrosis.

The cause for disappearance of the tumor at times is probally due not to the toxic action of predonin on the tumor cells, as with anticancerous drugs, but probably because of interference with the protein metabolism, inhibiting the proliferation and growth of the sarcomatous tissue and causing hyalinization and fibrosis.

AAN, a synthetic lathyrus factor, which exerts considerable effect on the bone, does not seem to effect changes in this sarcoma. This probably is due to the difference in effects on the normal bone and the proliferating tumorous tissue. In the effects of these various substances on the sarcoma, osteoplasia was not observed. We wish further to observe the effects on the sarcome which may be caused by varying dosages and by other methods and also to study the changes which may be caused by other substances and under varying conditions.

Summery

1. Successive transplantations of experimental osteogenic sarcoma in albino rats into the subcutaneous tissue of the backs of the same strain of albino rats were done.

2. The changes in the histological picture of this sarcoma during the course of transplantations were studied and the following results were observed. If the immature portions of the transplanted sarcomas are used successive serial transplantations are possible. However, at times, fibrosis, osteoplasia, and giant cell tumor like tissues were seen.

3. X-ray radiation, anticancerous drugs, predonin, and AAN were administered and their effects were observed. In the cases of X-ray radiation and anticancerous drugs, atrophy of the tumor cells and necrosis were distinct. In the anticancerous drug cases, the foci of necrosis were circumscribed. In predonin administered group, atrophy and fibrosis were conspicuous. In AAN administered group, almost no change was observed.

The authors are indebted to prof. W. Maeda and Prof. T. Kobayashi for valuable sugges-

tions and advice.

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Explanations of Plates XV—XVIII

Fig. 1 Gross appearance of the sarcomatous growth, 3 weeks after transplantation into the back of an albino rat, showing irregularly spherical shape with firm elastic consistency. The animal becomes anemic, cachectic and dies.

Fig. 2 Photomicrograph of the peripheral portion of the tumor of Fig. 1, showing immature cell groups. The cells with slight variation are medium in size and are clustered together to form lobules, around which blood vessels or loose connective tissue exist. In this stage of the tumor growth, osseous tissue has never been found.

Fig. 3 Photomicrograph of tumor cells, showing formation of lobules or funiculi between which blood sinuses are noted. In this type of tissue is often observed afterwards a process in bone formation in the primary bone sarcoma.

Fig. 4 Photomicrograph of the sarcomatous tissue, showing tendency toward fibrosis.

Fig. 5 Photomicrograph showing the appearance of osteoplasia in firoplastic sarcoma after six generations of successive transplantation.

Fig. 6 Photomicrograph of the sarcoma showing many giant cells and resembling human giant cell tumor.

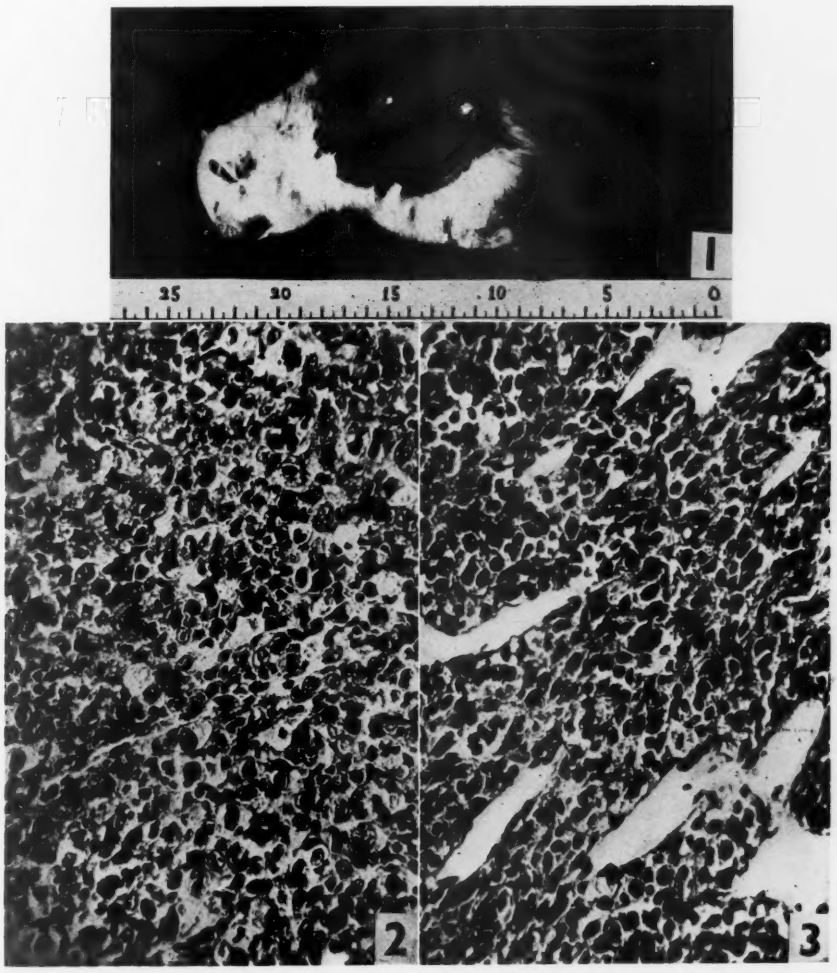
Fig. 7 Photomicrograph showing histological picture of an immature sarcoma of first generation of Text-Fig. 2.

Fig. 8 Photomicaograph of the sarcoma of the fifth generation of Text-Fig. 2, showing intense atrophy, vacuolization, and increased intered intercellular spaces. These changes are no longer localized, but are spread diffusely.

Fig. 9 Photomicrograph of the sarcoma radiated with 250r, showing some dissociation of cells and edematous fluid in the intercellular spaces although small in amount.

Fig. 10 Photomicrograph of the sarcoma radiated with 1500 r, showing intense atrophy and dissociation of the tumor cells. The cytoplasm is small in amount and the nuclei are elongated.

Fig. 11 Photomicrograph of the sarcoma treated with Tespamin, showing relatively localized necrotic focus. In the necrotic focus, loss of nuclei of the tumor cells and residue of the decomposition of nuclei and cytoplasm are observed.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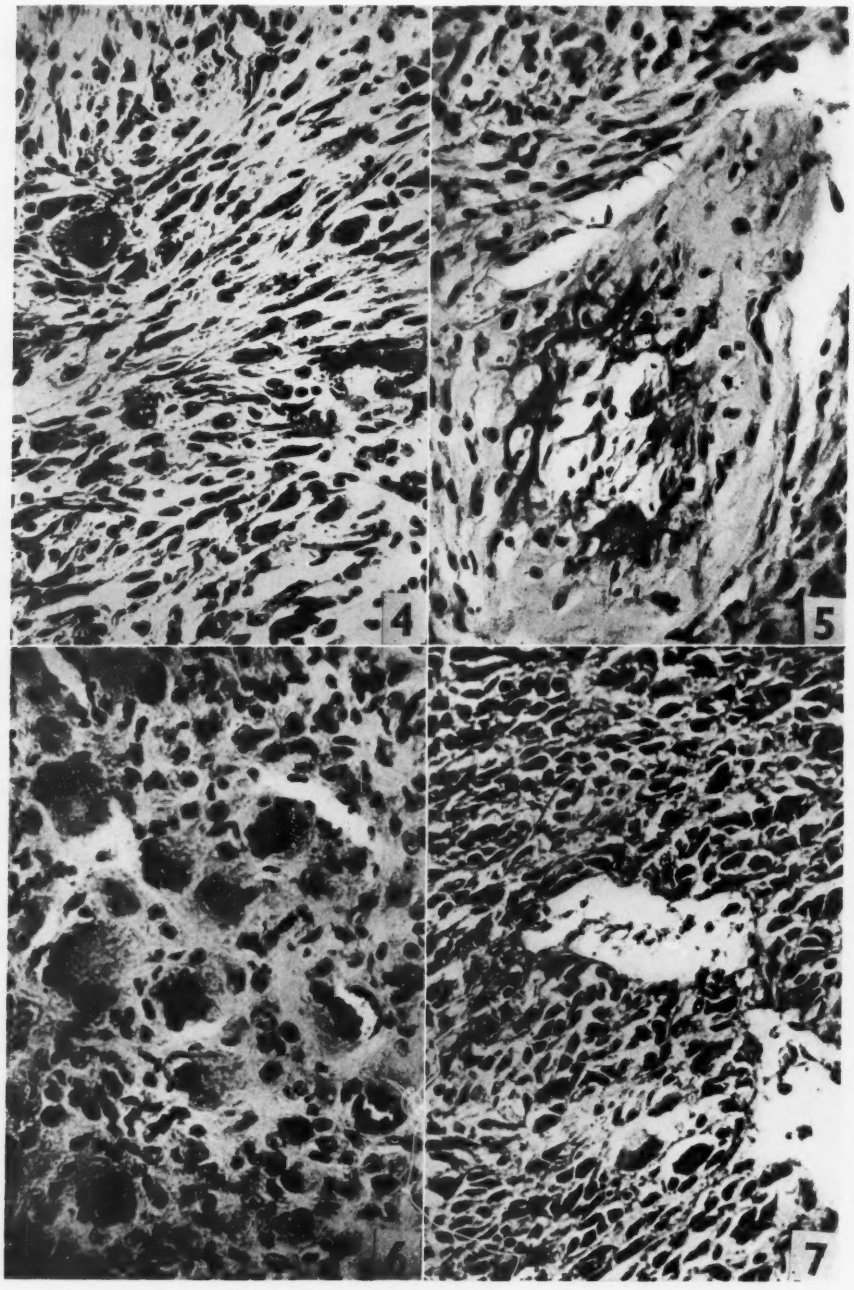
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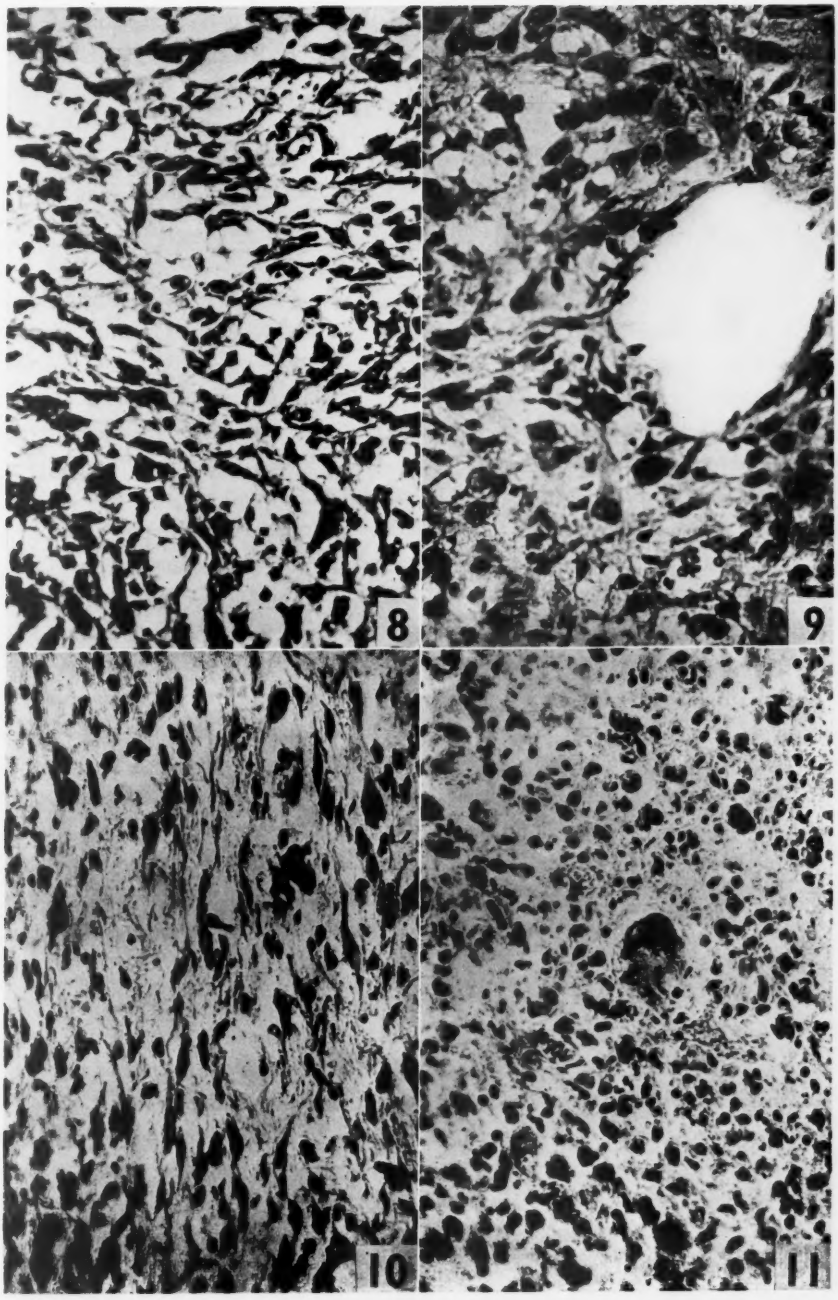
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[GANN, 52, 233~237; September, 1961]

A NEW TRANSMISSIBLE LEUKEMIA IN RF-STRAIN MICE

(Plates XVIII-XIX)

SHIGEYUKI OSAMURA, KENJIRO ITO, TOSHIO UEDA,
KYU SHIHO, SUMIO HATSUSHIBA and NAKAJI ITO

(Department of Internal Medicine, Tokyo Medical College)

Recently, Ehrhart and his coworkers succeeded in inducing myeloid leukemia in RF-strain mice after repeated subcutaneous injections of 1% indol-alchohol solution, the total amount of indole reaching 20 mg. Six months after the last injection, the incidence rate of leukemia was 15% of all the treated mice. In this leukemia, the leukocyte count increased to over 50,000 and there were over 60% leukemic cells in the peripheral blood. There was also marked extramedullary hemopoiesis in liver and spleen, and apparent infiltration of leukemic cells into kidneys and lungs. More recently, they reported that they were able to tlansplant this myeloid leukemia into other mice of the same strain by intravenous injection of the leukemic blood and by intraperitoneal injection of the homogenized spleen and liver of the diseased mice. In mice of the age of 4 weeks, the leukemias appeared after a latent period of 160–340 days subsequent to transplantation, and as early as 40–80 days after transplantation when younger animals were used.

We also have conducted the same experiment of leukemia induction by repeated injections of 1% indol-alchohol-solution using RF-strain mice, and have obtained one mouse showing leukemia of lymphatic nature, and have transplanted this leukemia at higher rate and in shorter period into other mice of the same strain by using the blood, spleen and lymph nodes of the leukemic mice. We also transmited this leukemia by using Seitz filtrate of the homogenized tissues.

We report here some findings on this new transmissible leukemia of RF-strain.

Materials and Method

As experimental animals we used RF-strain mice, whose parents were given to us by Prof. Langensorff of Freiburg, Deutschland, and which have been inbred in our laboratory. Three groups (each containing 10 mice) were treated with subcutaneous injections of 1 mg indol daily. We found one leukemic mouse in one group and this leukemia we have transplanted for ten generations by intravenous injection of 0.1 ml of leukemic blood and intraperitoneal injection of 1 ml of cell suspension of homogenized spleen and lymph nodes which was diluted to about 10% with physiologic

saline solution. We have also tried transmission with Seitz filtrate of homogenized leukemic spleen.

The Original Leukemia Mouse

During the daily 1 mg indol-injections into male RF-strain mice of the age of over 6 months, we found one mouse showing a leukemia picture of the peripheral blood after injections of 17 mg indol. The leukocyte count of the mouse showed 19.800/cmm, when indol doses reached 13 mg and 104,000/cmm when the doses reached 17 mg. Increased leukecytes showed over 60% of leukemic cells and on the following day the mouse died. Autopsy was conducted (Fig. 1).

At death, there were marked anemia and leukocytosis, i.e., red cell count: 6,010,000, hemoglobin: 80% and white cell count: 76,000.

In the blood findings of the smears we found 60% large lymphocytes and some percentage of immature lymphocytes, presumably lymphoblasts (Fig. 2). These lymphoid cells showed negative oxidase and peroxidase staining, poor staining by neutral red and negative phagocytosis of the tush granules.

Macroscopic finding of the leukemic mouse were as follows; The liver, the spleen, the lymph nodes and the thymus were markedly enlarged. The weight of the spleen reached 1.8 g (normal value: under 0.2 g), and that of the liver 2.2 g (normal value: under 1.5 g). The smears of the organs showed infiltration of immature lymphocytes. Microscopic findings of these organs were as follows; The trabecula of the spleen were atrophied and the follicle and sinus were markedly infiltrated with leukemic cells. Megacaryocytes were also seen in great numbers, pointing to active extramedullary hemopoiesis. The capsule showed no changes but in the subcapsular space there was cell infiltration (Fig. 3). The liver showed intensive infiltration around the vena centralis, sinusoids and Glisson's tissues, and the liver parenchyme cells were atropyied and fell into degeneration (Fig. 4). In the lymph nodes the original structure of the trabecula and the sinus were destroyed and replaced by the infiltration of many proliferated mononuclear cells. In the subcapsular space there was also seen infiltration by the same type of cells. The production of the reticular fiber was small (Fig. 5). The bone marrow cells were replaced totally by all sorts ot lymphocytes (Fig. 6). The thymus was markedly enlarged and intensive infiltration was seen in all portions of the organ (Fig. 7). In the interstitium of the kidneys, i.e., around the glomerulus and the tubulus, there existed intensive infiltration by the leukemic cells, there occasionally occurring hemorrhage in the tubulus (Fig. 8). In the lungs, slight infiltration was seen around the bronchiolus and vasculas.

Transplantation Experiments

In attempting to transplant this leukemia into other mice, we succeeded in this

transplantation in about 80% of the mice, which were inoculated with 10% cell suspension of leukemic mouse spleen. In positive cases the leukocyte count began to increases about 10 days after inoculation, reaching its peak after 3 weeks, that is with the leukocyte count 21,000, and then began to decrease and on 27th day the mouse died showing anemia and slight leukocytosis (40,000). The abnormal cells replaced a major portion of the normal mouse cells.

The histologic findings of the transplanted mouse leukemia were the same as the original mouse leukemia, that is, it showed marked enlargement of the spleen, liver, lymph nodes and thymus.

In analysing the data of the development of leukemias inoculated with the blood and the tissues of spleen and lymph nodes of the leukemic mouse, the period of induction shows a range of 3 to 45 days and 50% of mice are induced within about 14 days. The positive transmission was judged from the leukocytesis of over 20,000/cmm in the peripheral blood. The survival time of leukemic mice showed a range from 10 to 38 days and 50% of the mice died within 18 days. In other words, the mean latent period was 14 days and the mean survival time 18 days.

As for the difference in tne transmission rate according to the organs, positive transmission with blood was 90% ot the mice, which was the highest, followed by the homogenized spleen which showed the rate of 74.4%, and then by the lymph nodes which showed the rate of 46.0%. The total positive rate reached 70.8%. Regarding the difference in the positive rate according to age, the rate in mice within 6 weeks after birth showed a mean of 75.7%, and that in mice over 6 weeks after birth, 67.0%. The former showed better transplantability. But it is not necessary to use newborn mice, contrary to Gross' virus leukemia which can be transmitted by inoculation only to the newborn mice. The induction period after inoculation depend greatly upon the transplanted cell quantity. On the study of the induction by blood a cell quantity under 10,000,000/0.1 ml. showed a rate 80%, while cell quantity of over 15,000,000/0.1 ml. showed a rate of 100%, as judged 4 weeks after inoculation. And earlier inductions are possible by large numbers of cells. There are no differences between the induction rate according to sex.

Filtration Experiments

We could induce the same leukemia by injecting the Seitz filtrate of the homogenized spleen of the leukemic mouce (Table 1). Therefore, we consider the cause of our transmissible leukemia of RF-strain mice as a virus or a virus-like agent. This virus agent

Table 1. Induction by Cell-free filtrate. (observed 7 weeks after inoculation)

Cell-free filtrate		Cell suspension of spleen	
No. of Inoculation	No. of Induction	No. of Inoculation	No. of Induction
9	8	8	5
6	6	5	5
4	4	4	4

is hard to transmit to dd mouse. We are now studying the relationship between this virus and leukemic cell growth.

Discussion

Pointing out that indol is derived from tryptophan, Ehrhart emphasized that leukemia can be induced by the endogenous substanced in mice. Gross attributed the X-ray induced mouse leukemia to the activation of the parasitic virus in the mice. In our opinion we cannot overlook the possible activation of latent parasitic virus in our mouse leukemia. At any rate, we obtained the new transmissible leukemia in RF-strain mice which is probably due to virus agent. The induction of transplantable leukemia of Ehrhart *et al.* required a long period after inoculation and showed a low rate of induction, and they could not succeed in inducting leukemia by means of the Seitz filtrate of the tissue of leukemic mice. We are able to transfer our leukemia to other mice by using Seitz filtrate of the spleen and the lymph nodes of leukemic mouse, and we consider the cause of this leukemia to be a parasitic virus of the RF-strain mouse.

Ehrhart *et al.* reported that their indol-induced leukemia was that of myeloid type, but our leukemia seems to be that of lymphatic type.

Our leukemia which has been transmitted from one generation to another is considered by us from the standpoint of hematological and histological findings to resemble Friend's viral leukemia. Friend's virus leukemia or Friend's desease, however, is induced in Swiss mice by a virus isolated from Ehrlich's ascites carcinoma. The survival time of these leukemic mice is longer than that of ours, and the mean survival time is reported to be over 70 days. These leukemic mice showed more marked enlargement of the spleen with weights of 2–8 g and most of them died of rupture of the enlarged spleen. The type was that of the lymphatic leukemia, but at the same time there was marked erythroblastosis.

Therefore, we believe that we have established a new virus induced transmissible leukemia in RF-strain mice, and are still conducting experiments in the transplantation of this virus. Further reports will follow.

Grateful acknowledgment is made to Prof. Reiji Mizuno and Prof. Yuji Otaka of Department of Pathology of our Medical college for their helpful suggestions and assistance in preparing this manuscript.

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Explanation of Plates XVIII and XIX

Fig. 1.; Primarily induced lymphatic leukemia of Rf-strain mouse by Indol injection. Enlarged spleen and liver are seen.

Fig. 2.; Peripheral blood picture of leukemic mouse. Large lymphocytes are seen. ×1000.

Fig. 3.; Section preparate of the spleen of leukemic mouse. In the center of the picture megaczryocyte is observed. This means active hemopoisis in the spleen. ×500.

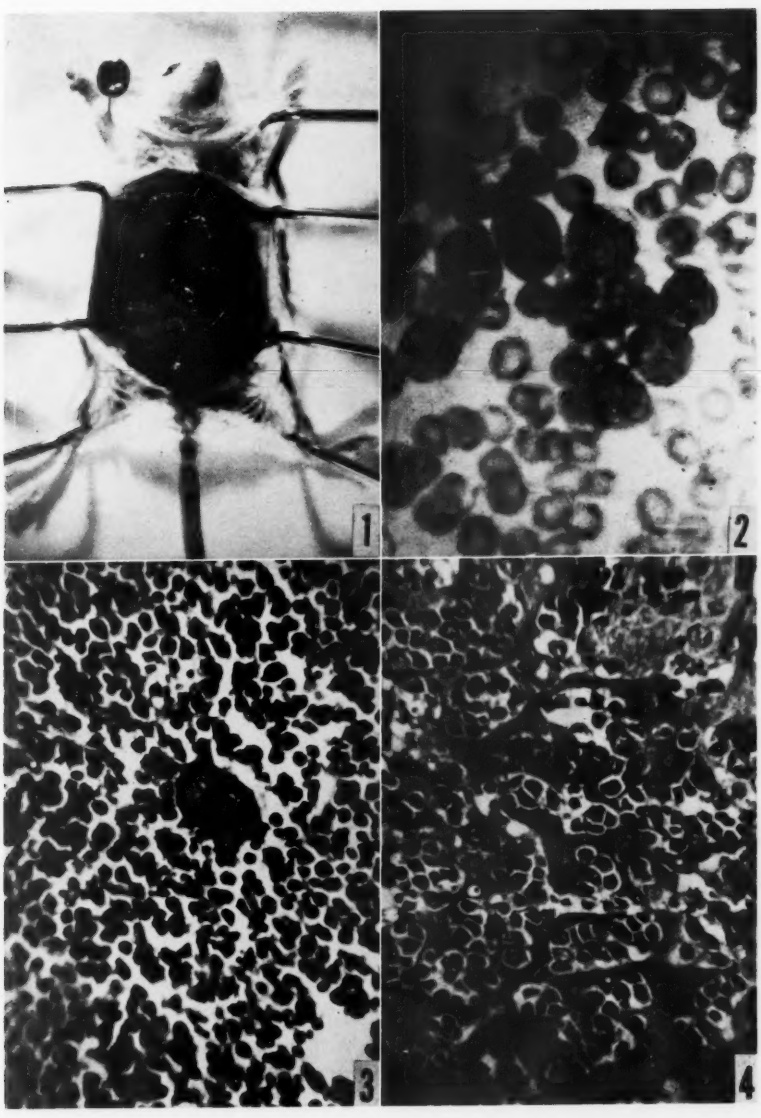
Fig. 4.; Section preparate of the liver of leukemic mouse. Sinusoids are occupied with large number of leukemic cells and liver parenchym cells are fallen into degeneration. ×500.

Fig. 5.; Section preparate of the lymph nodes. All portions of the organ are filled with leukemic cells. ×500.

Fig. 6.; Section preparete of the bone marrow of the femur. Leukemic cell infiltration is seen. ×500.

Fig. 7.; Section preparate of the thymus. There is seen leukemic cell infiltration. ×500.

Fig. 8.; Section preparate of the kidneys. Around the glomerulus and the tubulus, there are seen leukemic cell infiltration. ×500.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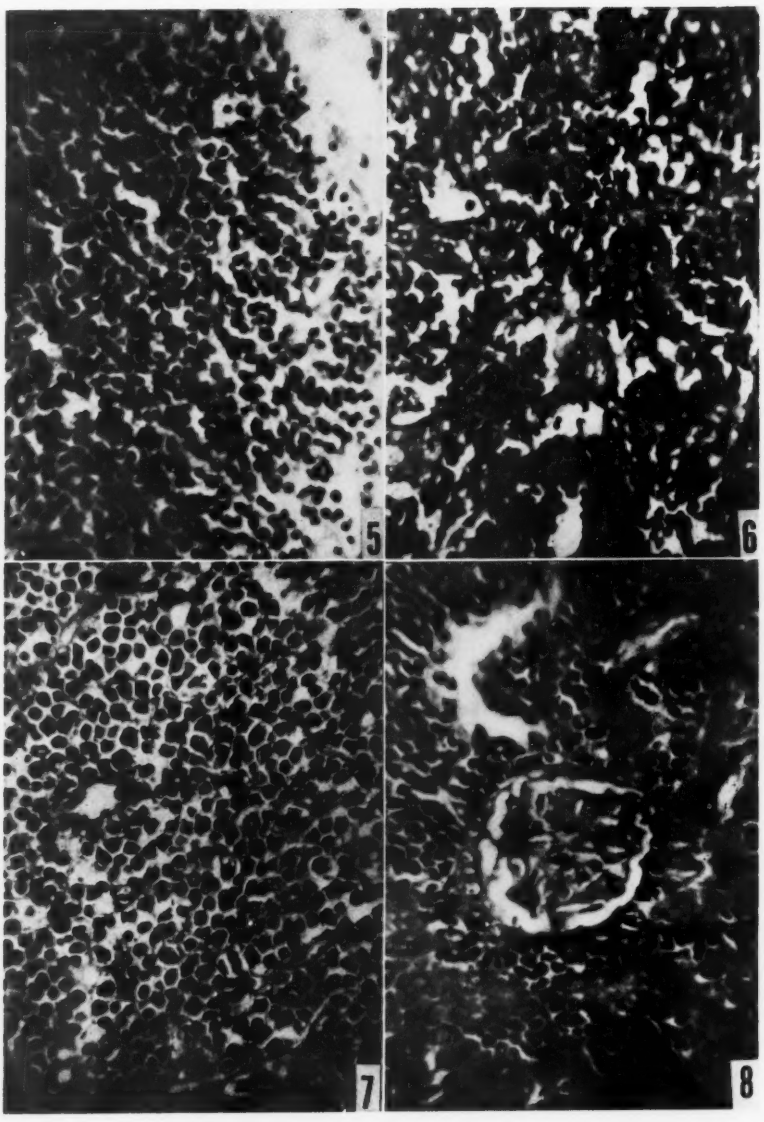
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[GANN 52, 239~245; September, 1961]

STUDIES ON THE MECHANISM OF LIVER-CARCINOGENESIS BY CERTAIN AMINOAZO DYES. V. N-DEMETHYLATION OF VARIOUS AMINOAZOBENZENE DERIVATIVES BY RAT LIVER HOMOGENATE WITH RESPECT TO THE CARCINOGENIC POTENCY

MITSUO MATSUMOTO and HIROSHI TERAYAMA
(Department of Biophysics and Biochemistry, Faculty of Science University of Tokyo)

Among many experimental animals, only rats and mice are known to be sensitive to the hepatocarcinogens such as DAB (1) and 3'-Me-DAB (2). It is very interesting to think about such remarkable species or tissue specificity in the chemical carcinogenesis demonstrated by aminoazo dyes and it may be hoped that research of the mode of such specificity may give a clue to the biochemical mechanism of the carcinogenesis induced by the aminoazo dyes.

The facts that only rats and mice are the animals able to decompose administered aminoazo dyes most vigorously (3)* and that among tissues of the sensitive rat, liver possesses the strongest metabolizing ability (3) (4) against dyes may probably be interpreted as indicating the intervention of the dye metabolism in the carcinogenic mechanism.

If we take an idea that one or some intermediate metabolites of the administered aminoazo dye are the actual carcinogen(s) responsible directly for carcinogenic reactions or changes of the liver cells, it is of course necessary to know the kinds of metabolisms concerned and the interactions of the metabolites with the cellular components.

The primary metabolic reactions of aminoazo dyes include hydroxylation of aromatic rings, reductive cleavage of the azo bond and oxidative N-demethylation at position 4 amino group. The metabolic intermediates produced by these primary metabolisms will be further metabolized and finally be excreted. If we consider that only a few intermediate metabolites are responsible for the initiation of cancerogenic changes in the liver cells, the other metabolisms which do not give rise to such carcinogenic intermediates and which decomposed then into nontoxic or excretable substances may be considered antagonistic metabolisms. It is then obvious that the interrelation between the carcinogenic and the antagonistic metabolisms should be taken into consideration for the complete understanding of the carcinogenic mechanism.

* Human liver (biopsy) did not show appreciable metabolic activity against DAB under the same conditions employed for rats and mice liver. (Unpublished data by Terayama).

As one of the ways aprroaching this goal, the comparison of various metabolisms of aminoazo dyes having different carcinogenic grades seems to be useful. A slight structural modification of the dye is known to change the carcinogenic potency considerably. It is very interesting to investigate how such a structural modification is reflected on the metabolism of the dyes. Series of experiments has been commenced with the above idea. In this paper N-demethylation of various aminoazo dyes was measured and compared with their carcinogenic potencies.

Experimentals

Aminoazo Dyes: 4-dimethylaminoazobenzene (DAB), 4-monomethylaminoazobenzene (MAB), 4-aminoazobenzene (AB), 3-methyl-4-dimethylaminoazobenzene (3-Me-DAB), 2-methyl-4-dimethylaminoazobenzene (2-Me-DAB), 2′-methyl-4-dimethylaminoazobenzene (2′-Me-DAB), 3′-methyl-4-dimethylaminoazobenzene (3′-Me-DAB), 4′-methyl-4-dimethylaminoazobenzene (4′-Me-DAB), 4-methyl, ethylaminoazobenzene (Et-MAB), 4-methyl, *n*-propylaminoazobenzene (*n*-Prop-MAB), 4-methyl, *iso*-propylaminoazobenzene (*iso*-Prop-MAB), 4-methyl, hydroxyethylaminoazobenzene (HOEt-MAB), 4-methyl, benzylaminoazobenzene (Benz-MAB), 4-diethylaminoazobenzene (Et, Et-AB) were employed for the study. Some of the dyes were prepared by Dr. A. Hanaki and by Dr. J.A. Miller, and the rest of the dyes were purchased or prepared by the authors. All dyes were purified by the repeated alumina column chromatography before use.

Enzyme Material and Preparation: Rats (Wistar, male 100–150 g weight) were administred with 5 mg of 3-methylcholanthrene dissolved in 0.5 ml corn oil by injecting intraperitoneally in order to increase the N-demethylase activity of the liver (5). Fourtyfive to fifty hours later the rats were killed by decapitation and the liver was perfused with 1.15% potassium chloride *in situ*. The fresh liver was then homogenized in 1.15% KCl by a Potter-Elvehjem homogenizer at 0°–4°C. Twenty per cent liver homogenate thus prepared was used for the N-demethylation study immediately after the preparation.

Reaction Condition of N-Demethylation: As the enzyme material, the above described rat liver homogenates were used. The N-demethylase which is located in the microsome fraction of the liver homogenates requires reduced form of triphosphopyridine nucleotide and molecular oxygen (6). As a TPNH generating system, glucose-6-phosphate and its dehydrogenase system was used in this case. The composition of the reaction mixture is shown in Table 1. The mixture was incubated at 37°C for 30 minutes and shaken gently during it. The vessel was open to the air for the supply of oxygen.

Measurement of N-Demethylation: Quantities of formaldehyde produced during the incubation of 30 minutes were determined by a modified MacFadyen's method**.

** An improved technique of the MacFadyen's formaldehyde determination by M. Matsumoto (shortening the heating time to 5 minutes).

Table 1. Composition of the Reaction Mixture for the Enzymic N-Demethylation.

Components	Concentration	Volume (ml)
TPN	1 mg/ml	0.1
DPN	1 mg/ml	0.1
Glucose-6-phosphate	0.01 M	0.3
$MgCl_2$	0.1 M	0.2
KCl	0.2 M	0.2
Nicotinamide	1.0 M	0.2
Semicarbazide	0.2 M	0.2
Phosphate Buffer (pH 7.4)	0.15 M	1.0
Distilled Water		0.6
Homogenate (in 1.15% KCl)	20%	1.0
Aminoazo Dye (in 95% EtOH)	10 mM	0.1
		Total Volume 4.0 ml

The reaction was stopped by adding 2 ml of 20% trichloroacetic acid (TCA) and the precipitates were removed by centrifugation. An aliquot of 0.5 ml of the supernatant containing formaldehyde was mixed with 4.5 ml of the chromotropic acid-H_2SO_4 reagent of Mac Fadyen (7) (1 g chromotropic acid dissolved in 100 ml distilled water, then diluted to 500 ml with 12.5 M H_2SO_4). The mixture was heated in a boiling water bath exactly for five minutes and then cooled in running water. The color developed was measured at 570 mμ using a Beckman spectrophotometer model DU. As the blank test, the same procedure was repeated without the substrate (aminoazo dye) and this solution was used as the reference for the above spectrophotometric measurement. Minor contributions to the absorbancy at 570 mμ by the aminoazo dyes were measured independently and were taken into consideration for the corrections. An empirical equation relating the corrected absorbancy to formaldehyde concentration in the chromotropic acid mixture was derived from a standard curve which was linear under the experimental condition.

$$E\ 570\ m\mu = 0.55C' \qquad (C' = [HCHO]\ \mu g/ml)$$

The aminoazo dyes were divided into three groups according to their structures as described in the following chapter. For dyes belonging to the same group, the same preparation of the homogenate was used.

Hepatocarcinogenic Potency of Aminoazo Dyes: The relative carcinogenic potencies of aminoazo dyes were taken from the data by Miller *et al.* (8). In the carcinogenic scale invented by Miller, the grade of DAB used as the standard is 6.

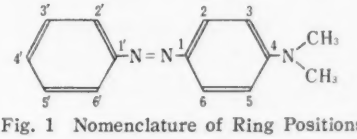

Fig. 1 Nomenclature of Ring Positions of DAB.

Results

N-Demethylation of Ring-monomethylated Derivatives of DAB: The carcinogenic potency of ring monomethylated derivatives of DAB differs greatly according to the position of the substitution.

The susceptibility of the dye to N-demethylase was expressed in term of amounts of formaldehyde formed during the incubation with the rat liver homogenate under the condition descrived above. The relationship between the N-demethylation and the carcinogenicity of the ring monomethylated derivatives of DAB is shown in Table 2. The potent carcinogens such as DAB and 3'-Me-DAB are demonstrated to be easily demethylated. The situation, however, is rather complicated with the non-carcinogenic aminoazo dyes. Non-carcinogenic 3-Me-DAB is hardly demethylated, but equally noncarcinogenic 2-Me-DAB shows rather a great tendency to be demethylated.

N-Demethylation of 4-N-alkyl Derivatives of MAB: The susceptibility to the

Table 2. N-Demethylation of Ring-monomethylated Derivatives of DAB and their Carcinogenicity.

Aminoazo-Dye	Formaldehyde Formation (μmole)	Relative Carcinogenic Activity
DAB	0.77	6
3-Me-DAB	0.27	0
2-Me-DAB	0.80	0
2'-Me-DAB	0.81	2-3
3'-Me-DAB	0.69	10-12
4'-Me-DAB	0.52	<1

Table 3. N-Demethylation of N-Alkyl Derivatives of MAB and their Carcinogenicity.

Aminoazo Dye (N, Me-R)	Formaldehyde Formation (μmole)	Relative Carcinogenic Activity
-N, Me-H	0.35	6
-N, Me-CH_3	0.72	6
-N, Me-CH_2CH_2OH	0.05	0
-N, Me-CH_2CH_3	0.39	6
-N, Me-$CH_2CH_2CH_3$	0.47	—
-N, Me-$CH\langle^{CH_3}_{CH_3}$	0.21	—
N, Me-$CH_2C_6H_5$	0.06	0
-$N\langle^{H}_{H}$	0.02	0
-$N\langle^{CH_2CH_3}_{CH_2CH_3}$	0.02	0

Table 4. Comparison of Ring-monomethylated Derivatives of DAB and MAB with Respect to the N-Demethylation and Carcinogenicity.

Aminoazo Dye	Formaldhyde Formation (μmole)	Relative Carcinogenic Activity
MAB	0.39	6
DAB	0.78	6
3-Me-MAB	0.44	<1
3-Me-DAB	0.27	0
3′-Me-MAB	0.44	10–12
3′-Me-DAB	0.81	10–12
p-N, N-Dimethyl-phenylenediamine	0.00	0

N-demethylase of a group of N-alkylated derivatives of MAB is shown in Table 3 with reference to the carcinogenic potency. The substitution of an alkyl group at the amino group appears to affect the rate of oxidation of methyl group attaching to the same nitrogen. Rather polar hydroxyethyl or benzyl group substitution reduces the N-demethylation rate considerably, but the substitution of linear alkyl groups seems to accelerate the N-demethylation in the following order: n-propyl> ethyl>(methyl)>hydrogen. The exceptionally large formation of formaldehyde from DAB compared with from MAB may be explained by the presence of twice as much N-methyl groups in DAB accesible to the N-demethylase. The substitution by isopropyl decreased the demethylation rate probably due to the steric mechanism.

From the data shown in Table 3, it may be remarked that the poor demethylation seems to be related to the weak carcinogenicity of the dye.

Comparison of N-Demethylation Between Ring-substituted Derivatives of DAB and MAB. As shown in Table 4, the rate of N-demethylation of DAB or its derivative is much greater (about twice) than that of MAB or its derivative except in the case of position 3 substituted derivatives.

As to the carcinogenic potency, no appreciable difference was observed between DAB and MAB or between 3′-Me-DAB and 3′-Me-MAB. The situation, however, is rather complicated with position 3 substituted derivatives. 3-Me-MAB showed the similar N-demethylation behavior to MAB or 3′-Me-MAB, but carcinogenic potency of the former was considerably low compared with the latter. With 3-Me-DAB, the demethylation rate was very small and the carcinogenicity was almost null. The substitution at position 3 seems to be very influential both on carcinogenicity and demethylation. The steric hinderance between 3-methyl and 4-dimethyl amino groups in the case of 3-Me-DAB has been clearly demonstrated by the spectral investigation (9). The decrease in carcinogenic potency of 3-methyl substituted derivatives of DAB and MAB is very interesting since this position was assumed res-

ponsible for the protein binding by Terayama *et al.* (10). According to the literature, 3, 5-diMe-DAB was also noncarcinogenic (9).

Discussion

The primary metabolic product of N-demethylation of the N-methylated aminoazobenzene derivatives by hepatic N-demethylase is formaldehyde, which is known as a strong cytotoxic substance (11) and which sometimes acts as a carcinogen (12). The formaldehyde produced in the tissue will be further metabolized and be largely excreted as CO_2 (13) probably via formic acid. It is a well known fact that formaldehyde reacts with proteins (14), nucleic acids (15) and other biologically important substances. A possibility was presented also by Terayama *et al.* that the bridge between the dye and liver proteins may be derived from active formaldehyde in *statu nascendi*. If the formaldehyde produced by the N-demethylase was not further metabolized quickly due to the high rate of the production or due to the weak formaldehyde metabolisms, genetically or pathologically controlled, the formaldehyde accumulates in the tissue and causes many troubles by the mechanisms mentioned above. If the damage by the formaldehyde is so severe that it may kill the cells, the liver necrosis may follow. Sometimes it becomes fatal to the experimental animals. Some of the numerous liver cells may not be damaged sufficiently to be killed. Out of such survived cells, regenerative proliferation may follow. In the course of the dye feeding, the regenerative proliferation takes place under the unphysiological circumstances. During this period most of the cells get adapted to the dye probably by the enhanced detoxicating metabolism against the dye (16). However, with only a small number of cells adaptation takes place through a different mechanism, i.e. irreversible loss of the enzymes responsible for the production of the toxic intermediate metabolites. Out of the latter cells, tumors may develope (16, 17).

Although we cannot say definitely at present that the N-demethylation is a principal carcinogenic metabolism concerned with carcinogenic aminoazo dyes, it may be said at least with safety here that aminoazo dyes hardly capable of generating formaldehyde are usually not potent carcinogens.

Summary

The N-demethylation rate of various derivatives of aminoazobenzene by rat liver homogenate was compared with each other in special respect to the carcinogenic potency. It was suggested that the poor N-demethylation was related to the weak carcinogenicity. Formaldehyde formed during the incubation was principally generated from N-methylated aminoazo dyes but not from N,N-dimethylphenylenediamine, a reductive product of DAB.

The role of formaldehyde in the carcinogenic mechanism was speculated.

Acknowledgment

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STUDIES ON CANCER INSUSCEPTIBLE ANIMALS I. INACTIVATION OF THE CARCINOGENICITY OF METHYLCHOLANTHRENE BY GUINEA PIG SERUM

TSUNEO BABA
(Cancer Institute, Japanese Foundation for Cancer Research, Tokyo)

INTRODUCTION

It has been well known that some species of experimental animals not only very rarely develop cancer spontaneously (1, 2, 3, 4), but also are resistant to artificial induction of cancer (5, 6, 7). The guinea pig stands out as a good example of such cancer insusceptible animal species.

It is a most fascinating fact that the guinea pig has not only a great resistance to a certain carcinogen but also to a variety of carcinogens. Namely, it is refractory to a series of carcinogenic hydrocarbons (5), as well as to aminoazo dye (6), to 4-nitroquinoline-N-oxide (8), etc. It is suggested also that it shows similar resistance to plastic film carcinogenesis (9) and to the carcinogenic virus factors.

If there is one common process in the mechanism of experimental cancer induction through a vast series of agents responsible, elucidation of the mechanism involved in the resistance to experimental induction of cancer in a certain species of animals should contribute much to the understanding of the mechanism involved in the carcinogesis in general.

Two different possibilities may be considered: either the guinea pig cells themselves are essentially insusceptible to malignant alteration, or the milieu surrounding the guinea pig tissue cells has an ability to inactivate or to neutralize carcinogenic factors.

The present experiment has been undertaken in the hope of clarifying the latter possibility. Sera from different species of animals with high and low susceptibilities to induction of cancer with hydrocarbons were allowed to react with methylcholanthrene (MC) *in vitro*, and the carcinogenic potencies of the mixtures were compared by injecting them subcutaneously into mice.

MATERIALS AND METHODS

Animal sera: Sera were obtained from 3 male adult rabbits each weighing ca. 1.5 kg, from 9 male guinea pigs each weighing ca. 500 g and from 20 male adult rats each weighing ca. 180 g, by bleeding from the carotic arteries. Cautions were taken

to get the sera aseptically. Sera from each species of animals were pooled and immediately used.

Incubation: 4.5 ml of serum fresh from one of the three species of animals was added to 0.5 ml of 2% methylcholanthrene (MC) solution in Tween 80 in a sterilized test tube. The test tube was shaked well by hand and incubated at 38°C for either 3 or 20 hours, after which the content was immediately used for the test of carcinogenic potency. During incubation frequent agitation was given.

Inoculation into mice: All the mice used for the assay of carcinogenic potencies were females of dd-strain kept on our standered diet and water *ad libitum*. In total 90 adult mice were divided into 6 groups of 15 mice each. Each of the MC-serum mixture after incubation was injected subcutaneously into the 15 mice in 0.1 ml doses. The injections were repeated once a week for 3 weeks at the same site in the lumbosacral region so that each mouse received 0.6 mg of MC in total.

The experiment was terminated at the 150th day after the first injection when the survivors were killed. All the animals were autopsied and diagnosis of every tumor was confirmed by histological examination.

Chemical analysis of the sera: Analysis was carried out in order to find out biochemical difference among the sera of those animals, if there is any. Total SH content of serum from each species of animals was assayed by the method of Flesch and Kun's colorimetric determination (10) after protein degradation, using equal volume of saturated urea solution. A/G ratio and total protein of serum were determined after Yoshikawa and Saito (11), and total cholesterol by the Zak-Henly's method (12). Paper electrophoretic analysis of the sera was also performed.

Results

As a few mice of every group were dead prior to the first sarcoma appearance, the data were evaluated by the effective mice which survived over 80 days. In all of the three groups, in which the serum-MC mixtures were injected after incubating for 3 hours at 38°C, sarcoma appeared in 100% in the experimental animals within 120 days. There seems to be no absolute inhibition of the carcinogenic potency of MC by any of the sera. However, as illustrated in Figs. 1 and 3, periods before the 100% appearance of sarcoma, were slightly different among the three groups. Namely, 100% of sarcoma incidence was obtained within 100 days in rat serum group, 110 days in rabbit serum group, and 120 days in guinea pig serum group.

After 20 hours of incubation, the serum-MC mixtures revealed striking differences in carcinogenic potency according to the species of animal from which the serum was derived (Figs. 2 and 4). In the rat serum group, subcutaneous sarcomas appeared in all the 13 mice which survived at the 110th experimental day, whereas 5 out of 9 mice (55%) in the rabbit serum group, and only 2 out of 9 mice (22%) in the guinea

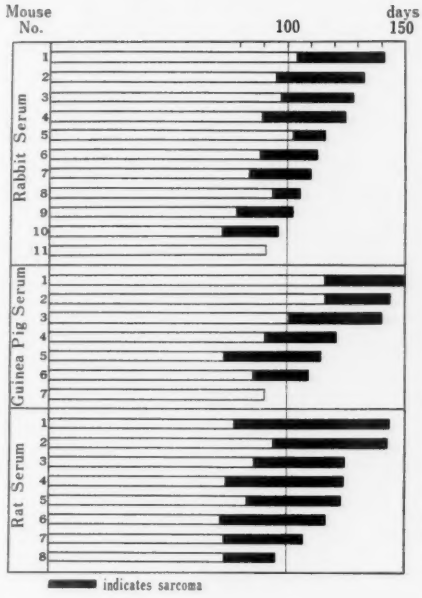

Fig. 1 Sarcoma production in mice by means of animal serum-MC suspensions which had been incubated in vitro for 3 hours at 38°C.

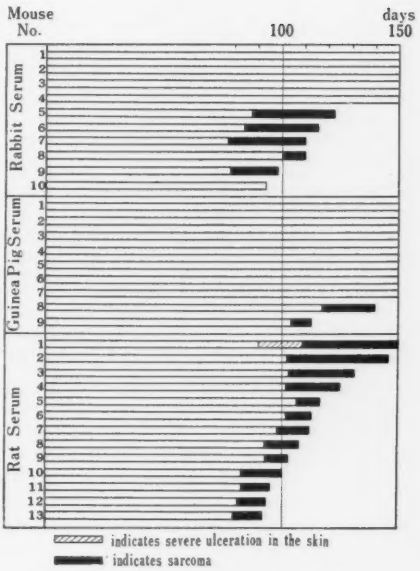

Fig. 2. Sarcoma production in mice by means of animal serum-MC suspensions which had been incubated in vitro for 20 hours at 38°C.

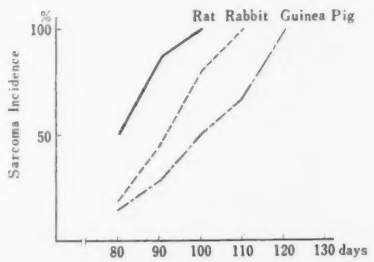

Fig. 3 Incidence of mouse sarcoma induced by animal serum-MC suspensions which had been incubated in vitro for 3 hours at 38°C.

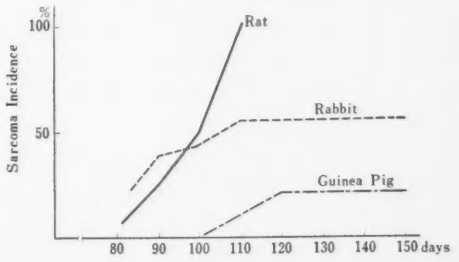

Fig. 4. Incidence of mouse sarcoma induced by animal serum-MC suspensions which had been incubated in vitro for 20 hours at 38°C.

pig serum group, developed sarcomas during the entire course of the experiment. The latency before the first appearance of sarcoma in each group is also distinctly different.

Table 1. Chemical Analysis of Animal Sera.

	Total SH* μg/ml	Total protein g/dl	A/G ratio	Cholesterol mg/dl
Rabbit	42–48	4.6	1.16	—
Guinea Pig	78–93	5.08–5.25	0.98–1.29	30
Rat	34–38	6.05–6.75	0.65–0.72	49

* Expressed as GSH equivalence.

It may be noted that guinea pig serum has a strikingly higher ability to inactivate the carcinogenicity of 20-methylcholanthrene *in vitro* than the sera from other experimental animal species. The chemical analysis of the sera (Table 1 and Fig. 5) revealed that the guinea pig serum differs markedly from rat serum in much higher SH content and in the characteristic electrophoretic mobility of α-globulin.

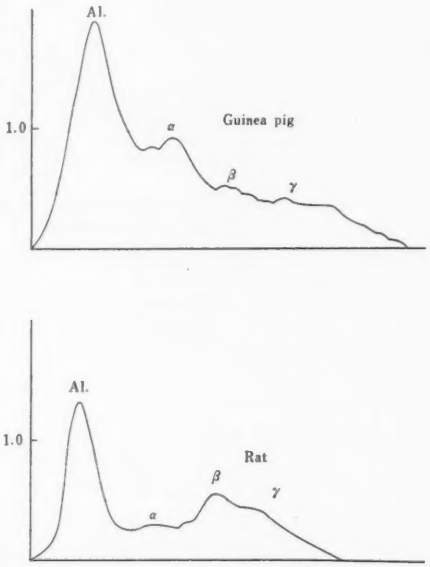

Fig. 5 Paper-electrophoretic diagrams of Guinea pig and Rat Sera.

Discussion

There has been some confusion about the difference in the susceptibility of variety of cells to chemical carcinogenic agents. In the chemical carcinogenesis, the potent substance concerned should reach and enter a somatic cell or cells where it can display the specific chemical activity. If there is a target in the cell itself the substance should act upon it through the intracellular milieu. The importance of the role both of the extra and intra cellular milieus, and the mechanism involved in the entrance of the substance into the cell has been often neglected.

It has been known that the somatic cells of the guinea pig can undergo malignant tumor formation under certain circumstances; Foulds, (13) Deals (14) and Biltris (15) have reported radiation carcinogenesis in guinea pig though low in incidence and long in latency, and Shimkin and Mider (16) successful chemical induction of malignancy in guinea pig if an extremely large amount of it was introduced. Thus, it appears that the guinea pig cells *per se* are not absolutely refractory to carcinogenesis, and that more attention should be directed to the characteristics of the internal milieu of the animal.

The present data indicate that the sera from different animal species exert different

degrees of inactivating effect upon the carcinogenic potency of MC, when injected into mice after 20 hours of incubation at 38°C with the chemical *in vitro*, and that this *in vitro* inactivation takes place most distinctly with the guinea pig serum. This suggests, in concomitance with the general insusceptibility of the guinea pig to tumor formation, that such inactivation may take place also *in vivo* more probably in the milieu than in the cells. Identification of a factor or factors present in the guinea pig serum and responsible for the inactivation would seem to be the next step in the study along this line.

There is an indication that tissue SH may play an important role in carcinogenesis (17, 18). In fact, some of the carcinogenic hydrocarbons (19, 20, 21) as well as carcinogenic 4-nitroquinoline-N-oxide (22) have been proved to react directly with SH. SH content in the guinea pig serum in the present experiment was definitely higher than those in the other animal sera (Table 1). However, it seems to be unlikely that the serum SH is solely responsible for the complete inactivation of MC in the present experimental system, even if all the available SH reacted directly with the MC; because the SH content of ca. 80 μg/ml in the guinea pig serum, expressed as GSH equivalence, means presence of only ca. 1.2 μ mole of SH in the whole 4.5 ml of the serum in a test tube system, agaist ca. 37 μ mole of 20-methylcholanthrene.

On the other hand, according to Mueller and Miller's experiment (23) unsaturated fatty acids must be considered as another possible biological material which would inactivate carcinogenic hydrocarbons *in vitro*. In the species differences in this connection, it has also been known that sheep sebum inactivates the hydrocarbon whereas human sebum does not (24, 25). Analysis of the content of unsaturated fatty acids in the sera is under way.

It is not clear likewise whether the difference of the electrophoretic mobility of α-globulin between the sera may or may not be involved in the present results. The guinea pig α_1-globulin is said to be a mucoprotein (26) and may be responsible for Kidd's mouse lymphoma inhibition phenomenon (27).

Still another possibility which should be considered is serum lipoprotein which is known to bind chemically with the hydrocarbons *in vitro* (28). The serum factor(s) responsible for the MC inactivation in the guinea pig, anyhow, remains to be determined.

Summary

Guinea pig serum was shown to have strikingly higher ability to inactivate *in vitro* the carcinogenicity of 20-methylcholanthrene than other animal sera. The mechanism involved in cancer insusceptibility of the guinea pig was discussed from the view point of this ability of its serum. The factor(s) responsible for the action in the

serum remains to be determined.

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[GANN 52, 253~256; September, 1961]

STUDY ON THE INTRAHEPATIC TOPOGRAPHICAL DISTRIBUTION OF PROTEIN BOUND AZO-DYE IN RAT BY MEANS OF C^{14} LABELLED P-DIMETHYLAMINOAZOBENZENE AUTORADIOGRAPHY

(Plates XX and XXI)

TSUNEO BABA

(Cancer Institute, Japanese Foundation for Cancer Research, Tokyo)

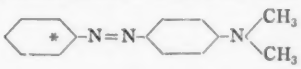
Since Miller and Miller, in 1947 (1), proved the presence of protein bound azo-dye in liver of rat fed carcinogenic azo-dye, and emphasized the importance of its role in the hepatocarcinogenesis, numerous authors have confirmed this and not a few important contributions to knowledge of carcinogenesis have been further submitted. Almost all of these investigations, however, have been from biochemical approaches and have been carried out with a material taken from the rat liver as a mass, without regard to the histo-topographical relations.

Though not all investigators on azo-dye carcinogenesis have always agreed with the theory that the protein binding plays an important role in the carcinogenesis, many biological characteristics of the bound dye seem to have formed very attractive subjects. On the other hand, Novikoff (2) and Shank *et al.* (3) have recently demonstrated histochemically as well as micro-biochemically that some of the enzymes are not always uniformly distributed in the hepatic lobules but show definite characteristic distribution therein.

The present experiment has been carried out using C^{14}-DAB autoradiography technique for the purpose of elucidating how the protein bound azo-dye is distributed topographically in the rat liver lobules and whether or not the bound dye is actually specifically found only in the liver cells but not in other components such as bile duct cells, Kupffer's cells and so on.

Materials and Methods

C^{14}-DAB: 1′-C^{14}-p-dimethylaminoazobenzene was used in the present experiment.



The specific activity of the compound was ca. 0.4 mc/mM. One hundred and ten mg of the compound was obtained by synthesis by Dr. Hanaki (National Inst. Radiological Science) from a 0.2 mc equivalent of 1-C^{14}-aniline which was imported from the Volk

Co., Ltd., U.S.A. (net weight 13.7 mg, specific activity 1.36 mc/mM).

Rat: A male albino rat of Wistar strain weighing 76 g at the beginning of the experiment was used.

Administration of the compound: Thirty mg of the C^{14}-DAB dissolved in 2 ml olive oil, was administered by stomach tube in two doses of 1 ml each in a day to the rat which was fasted for 12 hours beforehand. Then the rat was allowed to eat a 0.06% C^{14}-DAB rice diet *ad libitum* and to drink water freely as in the usual carcinogenesis experiment. The animal was killed by decapitation at the 73rd hour after the first force feeding, and was necropsied. All necessary organs were kept in acetone. For the liver, especially, each lobe was numbered, cut into pieces of a suitable size and a part of them was fixed with neutral 10% formol solution, and the rest was kept in acetone. The body and liver weights at the necropsy were 66 g and 4.0 g respectively. During the course of this experiment no abnormal disorder such as diarrhoea was noticed in the rat.

Preparation of histological specimen: Each liver lobe fixed in the neutral formol was rinsed with water for 48 hours, and dehydrated using ascending concentrations of alcohol for a relatively long period of time (about 7 days). The liver tissue was, then, passed through 2 baths of chloroform, 3 baths of paraffin solution, and embedded in paraffin as in the usual histological technique. Each paraffin block thus made was cut at 10 μ in thickness and the sections were floated on water and recovered on the slides, which were previously coated with gelatin, using 0.5% gelatin solution including 0.05% chromic alum, and dried.

Autoradiographic technique: The sections prepared as above were deparaffinized by usual laboratory procedures after dried enough. In order to remove free amino azo dye from the liver specimen, all the sections were repeatedly treated, for 20 hours at a time, with pure ethanol which was warmed at 60°C according to the original Miller and Miller's method (1). After that the sections were rinsed with ethanol of descending concentrations at room temperature and were finally kept in distilled water ready for stripping procedures. The slides thus prepared were covered by a Kodak AR-10 film under the usual stripping procedures, and kept in a special box made of lead at 5°C for suitable exposure periods.

Development and counter stain: After the exposures of 25 and 50 days the sections were developed according to the Kodak prescription and were counter-stained with Mayer's hematoxyline for 10 minutes and finally mounted in Canada balsam.

Results and Discussion

Intralobular topographical distribution of the protein bound azo dye in the rat liver has been shown as the grain distribution on the film.

As illustrated in plates, the grains were almost not observable in the regions

of bile duct systems. In the parenchymal hepatic cells, on the other hand, the C^{14} grains were numerously observable. Among the three zones of the lobule, peripheral, middle, and central, the distribution of the grain was almost uniform, except that seemingly slightly diminished tendency in the peripheral zone was found; differences in the radioactivity among the hepatic lobes were not essential as far as the present experiment was concerned. There was an evidence of some slight radioactivity in Kupffer's cells. Some radioactivity was proved to exist in a relatively large blood vessel, which may be caused by serum protein bound azo dye. The grains due to back ground in the present experiment was entirely negligible in the all sections examined. In the present experiment, the rat was killed at the 73rd hour after the first force feeding of C^{14}-DAB, according to Terayama *et al.* (4), who showed that the amount of bound dye in the liver reached maximum at this time under similar experimental conditions. Although some other authors (5) reported different time for the maximum binding, it must be noted that they did not give to the rat carcinogen-containing diet following the administration by the stomach tube.

That only the protein bound azo dye remained in the liver tissue sections after the treatment with the warmed ethyl-alcohol, was strongly supported from the fact that the expected radioactivity of the extracted liver section was proved to show almost equal counts to the calculated radioactivity of C^{14}-DAB bound protein isolated from comparable liver tissue.

Relatively uniform distribution of the protein bound azo dye in the hepatic lobuli elucidated in the present experiment may rationalize the biochemical studies hitherto reported as well as those to be done in the future. Attention should be paid, however, to the fact that protein bound C^{14}-DAB was found even in Kupffer's cells as well as in serum, though in relatively small quantities. The serum protein bound dye has recently been given attention by Dijkstra *et al.* (5), who have shown it to amount to almost half of the content in the liver of the same rat.

Summary

Intrahepatic, especially intralobular topographic distribution of the so-called protein bound azo dye in rat was studied by means of C^{14}-DAB autoradiography.

The distribution of the bound dye in the parenchymal liver cells was almost uniform among the three zones, peripheral, middle, and central, although there was a slight tendency to be less in the peripheral zone. Relatively small quantities of bound dye were proved to exist in Kupffer's cells as well as in the serum. The protein bound azo dye was scarecely detected in the bile duct systems.

Acknowledgements

The author's sincere thanks are due to Dr. Waro Nakahara and Dr. Kunio Oota for their stimulating encouragement, and to Dr. Hiroto Naora for his helpful discussion. He also wishes to thank Dr. Akira Hanaki, National Institute of Radiological Science, for his kindness in donating C^{14}-DAB.

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Explanation of Plates XX and XXI

Fig. 1. Peripheral zone of a lobule. Focused at film level. Exposure: 25 days. Magnification: ×1000.

Fig. 2. Peripheral zone of a lobule corresponding to Fig. 1. Focused at cellular level.

Fig. 3. Middle zone of the same lobule as Fig. 1. Focused at film level. Exposure: 25 days. Magnification: ×1000.

Fig. 4. Middle zone of the same lobule as Fig. 1, corresponding to Fig. 3. Focused at cellular level.

Fig. 5. Central zone of the same lobule as Figs. 1 and 2. Focnsed at film level. Exposure: 25 days. Magnification: ×1000.

Fig. 6. Central zone of the same lobule as Figs. 1 and 2. Corresponding to Fig. 5. Focused at cellular level.

Fig. 7. Peripheral and middle zone of a lobule. Exposure: 50 days. Magnification: ×400.

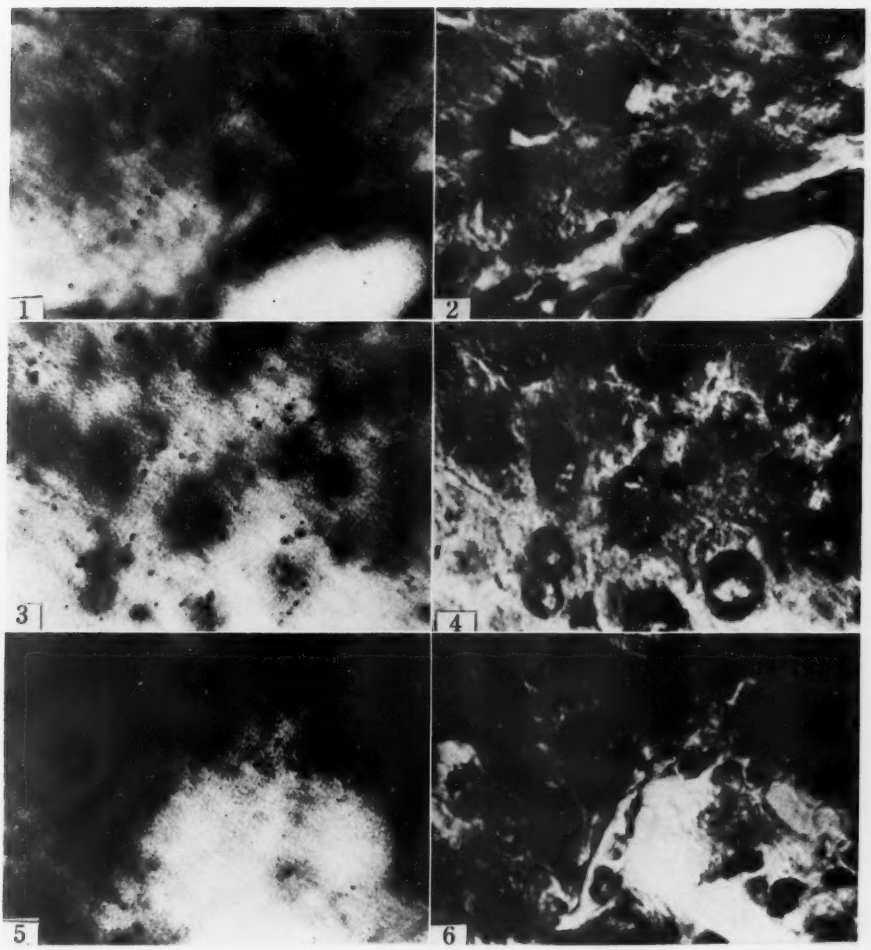
Fig. 8. Middle and central zone of the same lobule as Fig. 7. Exposure: 50 days. Magnification: ×400.

Fig. 9. Peripheral zone of a lobule. Exposure: 50 days. Magnification: ×1000.

Fig. 10. Middle zone of the same lobule as Fig. 9. Exposure: 50 days. Magnification: ×1000.

Fig. 11. Central zone of the same lobule as Figs. 9 and 10. Exposure: 50 days. Magnification: ×1000.

Fig. 12. An area of serum and blood cells in a large hepatic vessel. Exposure: 50 days. Magnification: ×1000.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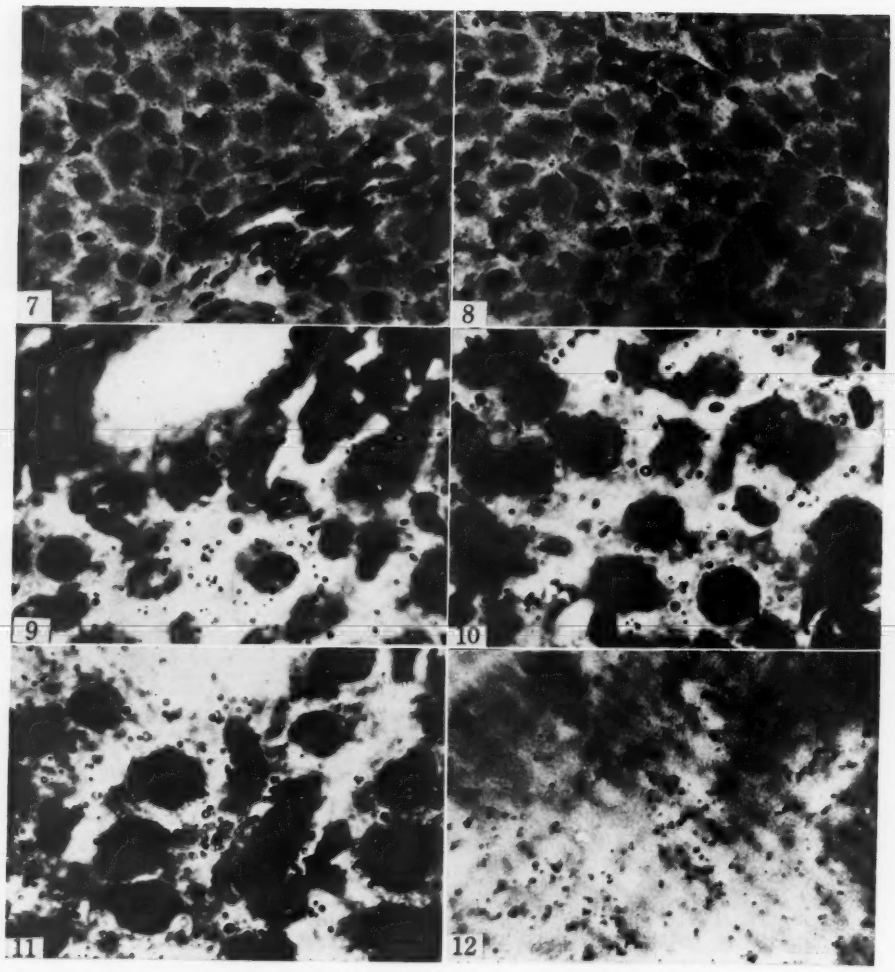
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[GANN, 52, 257~264; September, 1961]

CHROMOSOMAL ALTERATION AND THE DEVELOPMENT OF TUMORS, VII. KARYOLOGICAL ANALYSIS OF SPONTANEOUS AND INDUCED LEUKEMIAS IN MICE[1)2)]

YOSHINORI KURITA and TOSIHIDE H. YOSIDA
(National Institute of Genetics, Misima)

During recent years chromosomal studies have been carried out in leukemias of mice by several investigators by means of improved techniques and with special emphasis on the comparison of malignant with normal cells. Some investigators (Ford *et al.* 1958, Wakonig and Stich 1960, Stich 1960 and Hellström 1960) found that specifically different chromosome numbers in stem cells of leukemias developed either spontaneously or after treatment with radiation and chemicals. Stich *et al.* (1959) and Wakonig and Stich (1960), however, observed that somo spontaneous leukemias in AKR-mice contained normal chromosome types, but some others were characterized by a different chromosome pattern. Chromosomal similarity between normal and leukemic cells was observed by Bayreuther (1960) in various mouse leukemias.

The present study was undertaken to reinvestigate whether chromosomal alterations had taken place in 9 spontaneous AKR mouse lekemias and 3 radiation-induced leukemias in RF and C57BL mice.

Materials and Methods

Spontaneous leukemias: The spontaneous leukemias developed in AKR/JMs strain obtained from Roscoe B. Jackson Memorial Laboratory were used in the present study. The incidence of spontaneous leukemias in our laboratory amounted to about 90 percent (Yosida, Kurita and Sakamoto 1961).

Radiation-induced leukemias: C57BL/6Ms and RF/Ms strain mice were used for the study of radiation-induced leukemias. The former strain was sent by Dr. Heston of the National Cancer Institute, Bethesda, and the latter was obtained from Dr. Sugawara of the National Institute of Radiological Science, Japan. Susceptibility to radiation-induced leukemia was found in 35% of C57BL/6Ms mice and they were almost all of thymic lymphosarcoma type. They developed about 8 months after the irradiation (Kurita and Yosida 1961).

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2) Supported by a Grant in Aid for Fundamental Scientific Research from the Ministry of Education (No. 0434 in 1961).

X-ray irradiation conditions were as follows; 250 kVp, 15 mA, filters of 0.3 mm Cu+0.5 mm Al, 50 cm distance, 60.8 r/min. Total 600 r were given in four fraction each of 150 r every week and total-body irradiation was applied to C57BL strain mice. In RF strain mice, total 300r were given in two fractions and 0.03 mg of 20-methylcholanthrene were injected subcutaneously into each mouse after the second irradiation. The leukemia incidence was over 50 percent, the disease developing on the average 4 months after the first irradiation. Its type was generalized lymphatic leukemia.

Enlarged spleens of the leukemic mice were used throughout the experiments for the observation of chromosomes which was mostly carried out in primary leukemias. The cytological technique was as follows: Colcemid was injected into the tail vein of leukemic mice. Two hours after injection, a part of the enlarged spleen was finely chopped in a Petri-glass and pretreated with hypotonic solution for 30 minutes at room temperature and then stained with acetic orcein. Spleens of newborn mice in AKR/JMs, RF and C57BL/6Ms strains were used for the control, using the same technique as for the observation of leukemic chromosomes.

Results of Observations

1. Chromosome numbers

a) Spontaneous leukemias: Chromosomes in nine spontaneous leukemias developed in AKR strain were observed. They were named AKLA, AKLB.....in order of their development. Cells of AKLA leukemia developed in a femal mouse were characterized by 36-41 chromosomes with the mode at 40. The range of variation in this material was narrower than in the other leukemias (Table 1). The modal

Table 1. Distribution of chromosome numbers in spleen cells of nine spontaneous leukemias in AKR strain mice. (this table is shown by %)

Name of leukemia	No. of cells observed	Chromosomes numbers										
		≦35	36	37	38	39	40	41	42	43	44	45≦
AKLA	40	0	5.0	0	5.0	10.0	70.0	10.0	0	0	0	0
AKLC	111	9.1	0.9	7.2	10.0	6.3	15.3	41.3	7.2	0.9	0.9	0.9
AKLD	32	6.2	3.1	9.4	3.1	9.4	56.2	3.1	9.4	0	0	3.1
AKLE	40	5.0	0	0	15.0	10.0	65.0	2.0	0	0	0	0
AKLF	32	12.5	0	12.5	6.3	6.3	62.4	0	0	0	0	0
AASG	53	0	0	1.9	0	1.9	22.6	1.9	0	0	1.9	68.2
AKLH	30	0	3.3	3.3	3.3	0	16.6	53.3	13.3	0	0	6.6
AKLI	84	5.9	7.1	2.4	3.6	7.1	26.2	29.7	11.9	3.6	0	2.4
AKLJ	43	4.7	7.0	16.2	7.0	16.2	34.9	7.0	0	0	4.7	2.3
Control*	30	0	10.0	0	13.0	16.7	60.0	0	0	0	0	0

* Spleen tissue in newborn AKR-mice.

chromosome number in leukemia AKLC developed in a female mouse was 41 (41.3 %). As the table shows (Table 1) a wide range of variation was found in this case (35-45). Frequency of cells with 40 chromosomes ranked the second (15.3%). Three leukemias, AKLD, -E and -F, developed all in male mice, were characterized by the mode at 40. Range of chromosome number distribution in the AKLD was still wider than in AKLA and AKLC. Leukemia AKLG had a very wide range of variation of chromosome numbers, fluctuating from 32 to 87. It showed two modes one at 40 and the other, secondary mode, at the hypotetraploid number. Distribution of chromosome numbers in the tetraploid range was wider than in the diploid range (Table 2). Another leukemia again with the mode at 41 was AKLH, which developed in a female mouse. Tendency of chromosome number distribution was similar to that of AKLC. Chromosome counts in 84 clear metaphase plates in an AKLI showed that the chromosome number varied from 33 to 46 with bimodal distribution, one mode at 41 (29.7%) and the other at 40 chromosomes (26.2%). The last leukemia AKLJ had the mode at 40 and showed considerable fluctuation. Spleen cells in newborn mice were examined as control. The results of chromosome counts in 30 cells showed that the number varied from 36 to 40 with the mode at 40 (60.0%).

b) Induced leukemia : Chromosomes of a radiation induced leukemia in a C57BL mouse (♀) were analysed. The neoplasm was characterized by the modal chromo-

Table 2. Distribution of chromosome numbers in the polyploid variation range in leukemia AKLG.

Chrom. number	45-70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85≧
%	15.1	5.7	1.9	1.9	5.7	1.9	9.4	7.6	1.9	5.7	1.9	5.7	0	1.9	0	1.9

Table 3. Distribution of chromosome numbers in radiation-induced leukemias of C57BL and RF mice (%).

Name of leuk. strain	No. of cells observed	Number of chromosomes										
		35	36	37	38	39	40	41	42	43	44	45
C57BL	125	2.4%	0	6.4	5.6	9.6	45.6	17.6	3.2	3.2	1.6	4.0
RF (1)	40	0	0	2.5	0	0	7.5	45.0	45.0	0	0	0
RF (2)	30	3.3	0	3.3	3.3	0	16.7	66.7	3.3	3.3	0	0
Control of BL*	30	22.7	0	0	0	3.3	66.7	0	3.3	0	0	0
Control of RF**	30	11.0	0	0	3.3	10.0	76.7	0	0	0	0	0

* Spleen cells in newborn C57BL mice.

** Spleen cells in newborn RF mice.

Figs. 1–12. Camera lucida drawings of chromosomes in normal and leukemia cells in mice. 1, normal spleen cell of a AKR mouse (40 chromosomes). 2, leukemia AKLA cell (41 chromosomes). 3, leukemia AKLC cell (40 chromosomes). 4 and 5, leukemia AKLG cells containing 78 and 63 chromosomes, respectively. 6, leukemia AKLI cell (40 chromosomes). 7, normal spleen cell of C57BL mouse (40 chromosomes). 8, induced leukemia C57BL cell (40 chromosomes). 9, normal spleen cell of an RF mouse (40 chromosomes). 10, induced leukemia RF–2 cell (41 chromosomes). 11 and 12, induced leukemia RF–1 cells containing 42 and 41 chromosomes, respectively.

Table 4. Change of chromosome number distribution in a C57BL leukemia by successive transplantations.

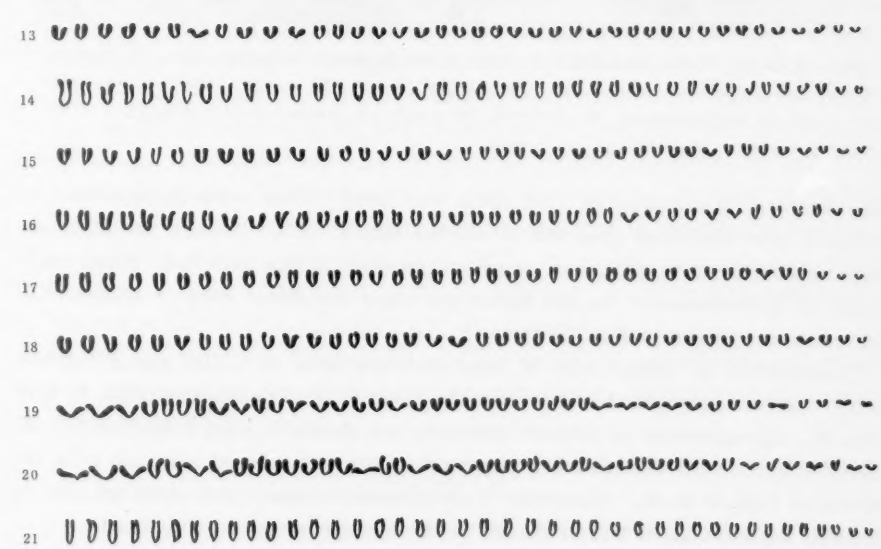
Transplant gener.	No. of cells observed	Number of chromosomes										
		35	36	37	38	39	40	41	42	43	44	45
1 to 3	91	2.2	0	6.6	3.3	11.1	35.4	21.2	3.3	3.3	2.2	6.6
26	34	3.0	0	5.8	11.8	5.8	64.6	3	3	3	0	0

some number at 40 as in most of the spontaneous leukemias observed (Table 3). It has been transplanted serially to C57BL mice for over thirty generations. It is noteworthy that 21.2% cells with 41 chromosomes were observed in the first to third transplant generations, while they were reduced to 3% in the 26th transplant generation, whereas the number of 40 chromosome cells was increased to 63% (Table 4).

Two leukemias were induced in RF strain female mice by use of radiation and chemicals. Of two leukemias one (RF-2) had the mode at 41 chromosomes and the other (RF-1) showed bimodal distribution with modes at 41 and 42. Range of the variation in RF leukemias was smaller than in C57BL leukemia (Table 3).

2. Idiogram analysis

Metaphase chromosomes in normal AKR-spleen cells and AKR leukemia cells are shown in Figs. 1 to 6 and 13 to 15. Although a variation of chromosome numbers



Figs. 13–21. Serial alignments of chromosomes in normal spleen and leukemia cells. 14, from Fig. 3. 15, from Fig. 6. 16, from Fig. 7. 17, from Fig. 8. 18, from Fig. 9. 19, from Fig. 10. 20, from Fig. 11. 21, from Fig. 12.

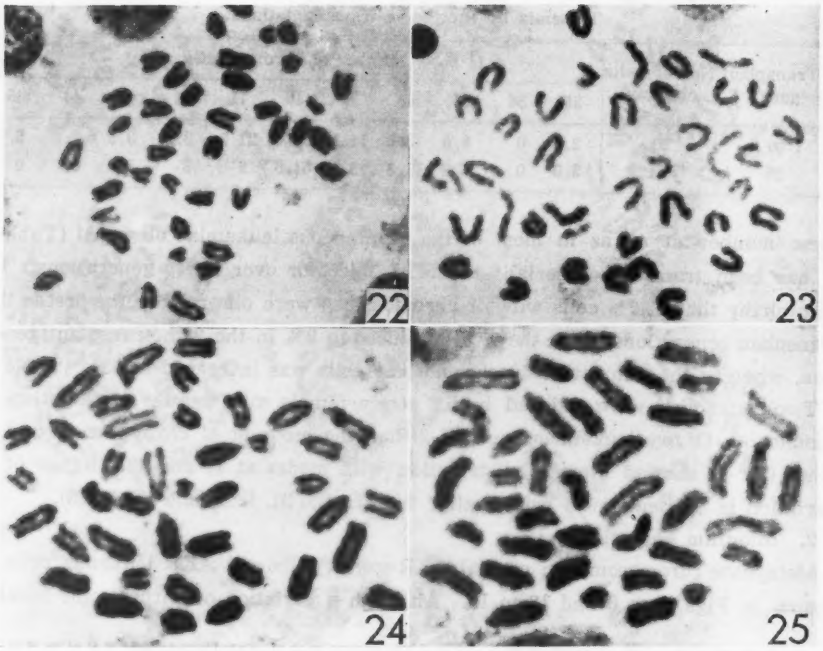


Fig. 22 to 25. Photomicrographs of chromosomes in mouse leukemia cells. 22. leukemia AKLD cell (40 chromosomes). 23. leukemia AKLG cell (40 chromosomes). 24. leukemia RF-1 cell (41 chromosomes). 25. leukemia RF-2 cell (41 chromosomes). Fig. 22. ×1800. Fig. 23. ×2000. Figs. 24 and 25. ×2200.

was observed, no alteration of their shape was found. They were characterized by terminal centromeres as observed in normal spleen cells. Marker chromosomes, such as a metacentric or minute, were not observed in this material. Serial alignments of chromosomes in normal spleen cells and neoplastic cells of some AKR-leukemias are shown in Figs. 13 to 15.

Chromosomes in spleen cells of male newborn mice of C57BL and RF strains were observed as control. They had the mode at 40 chromosomes (Figs. 7, 8, 16 and 18). Chromosomes in induced leukemias are shown in Figs. 8, 10–12 and 24–25. Serial alignments of chromosomes in normal spleen and induced leukemia cells are shown in Figs. 16 to 21. Alteration of chromosome number was observed also in induced leukemia cells, but no change of chromosome structure was found.

Discussion

Cytological studies on spontaneous leukemias of AKR mice have been carried out

by Stich *et al* (1959) and Wakonig and Stich (1960). According to them, among 16 mice with primary leukemias 10 had the modal chromosome number at 40 (normal diploid), 5 had the mode at 41 chromosomes and one animal had bimodal distribution with modes at 42 and 43. The results obtained by the present authors in AKR-mice were similar. We found that among nine AKR-leukemias 5 were characterized by the mode at 40, 2 by the mode at 41 and the remaining two by bimodal distribution. A spontaneous primary leukemia in a FAKI mouse was observed by Ford *et al.* (1958). They found a bimodal distribution of chromosome numbers with modes at 42 and 44. Chromosomes in four AK-mice with primary leukemias were observed by Bayreuther (1960). He found that all of them were characterized by normal diploid number, but three among them lost their karyotype integrity at the first transplant generation, and at the further transplant generations they showed a single specific abnormal cell type.

Based on the above investigation, it seems highly possible that the stem cells in spontaneous AKR-leukemias represent at least three types, namely (1) 40 chromosome type, (2) 41 chromosome type and (3) type with bimodal variation of chromosome numbers.

Chromosomes in radiation-induced leukemias in CBA mice were observed by Ford *et al.* (1958). In primary and transplanted leukemias they found various types of stem line cells regarding the modal chromosome number and occurrence of marker chromosomes. On the other hand, Bayreuther (1960) never found such chromosomal abnormalities in primary leukemias induced by radiation. The present authors' finding was that among three induced leukemias the modal chromosome number was in one at 40, in another at 41, and bimodal distribution, at 41 and 42 (RF-1) was found in the third one. Although the data for induced leukemias observed by the present authors are few, it is interesting that there occurred the same three types of variation in induced tumor stem cells as observed in spontaneous leukemias. Chemically induced leukemias were observed by Stich (1960) and Hellström (1960). All materials observed by them were characterized mostly by stem cells with 41 chromosomes and sporadic occurrence of 42 chromosomes.

From the comparative idiogram analysis in spontaneous and induced leukemias it can be concluded that the chromosomal situation was not different between spontaneous and induced leukemias.

Summary

Chromosomes in nine spontaneously developed leukemias in AKR strain were analysed. Among them five were characterized by the modal chromosome number 40; two had the mode at 41. The remaining two had a bimodal distribution of chromosome numbers, one with one mode at 40 and the other at 41, and the other

with one mode at 40 and the other at hypotetraploid 76.

Three strains of leukemias induced by X-irradiation were observed. The mode in one was at 40 (C57BL). Of two leukemias, developed in RF mice by double treatment with X-ray and 20-methylchoranthrene, one was characterized by the mode at 41 and the other by bimodal variation with modes at 41 and 42 chromosomes.

No marker chromosomes were found in all those leukemias, and no chromosomal difference was found between spontaneous and induced leukemias.

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